



Combination effects of pesticides on birth weight and metabolic programming in rat offspring

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**Ministry of Environment
and Food of Denmark**
Environmental
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Combination effects of pesticides on birth weight and metabolic programming in rat offspring

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Sources must be acknowledged.

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Foreword

The research presented in this report on developmental toxicity effects in experimental animals after mixed exposure to pesticides causing decreased birth weight was carried out from 2012 to 2015.

The project and the research were mainly financially supported by the Danish Environmental Protection Agency's Pesticide Research Programme (J. no. MST-667-00126). However, the assessment of some endpoints was also financially supported by another project supported by Danish Environmental Protection Agency. Overall, the authors wish to acknowledge the Danish Environmental Protection Agency for funding the project.

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A steering committee for the project was established and chaired by Jørn Kirkegaard until January 2015 and thereafter by Henrik Frølich Brødsgaard, the Danish Environmental Protection Agency (EPA)

We would like to thank the members of the steering committee for their involvement throughout the project period. Especially we want to thank the referees Henrik Leffers, Susanne Hougaard, Rikke Donchil Holmberg, and Martin Larsson for their constructive comments during the writing process of this report.

The studies presented in this report is published in the following two peer reviewed scientific papers. Some tables and figures have been updated in these papers.

Below you find reference for the two papers:

Hass, U., Christiansen, S., Axelstad, M., Scholze, M. & Boberg, J. Combined exposure to low doses of pesticides causes decreased birth weights in rats. *Reprod Toxicol* 72, 97–105, <https://doi.org/10.1016/j.reprotox.2017.05.004> (2017).

Svingen T, Ramhøj L, Mandrup K, Christiansen S, Axelstad M, Vinggaard AM, Hass U. Effects on metabolic parameters in young rats born with low birth weight after exposure to a mixture of pesticides. *Sci Rep*. 2018 Jan 10;8(1):305. <http://www.nature.com/articles/s41598-017-18626-x>

Summary and conclusions

Risk assessment of pesticides is generally based on the no observed adverse effect levels (NOAELs) for single compounds. However, humans are typically exposed to a mixture of several pesticides. For mixtures of endocrine disrupting chemicals including pesticides, experimental evidence show that substantial mixture effects on reproductive development can occur even though each of the individual chemicals is present at low, non-adverse doses.

Decreased birth weight is a common effect for many pesticides in experimental studies, but there are no data on the effects of combined developmental exposure to pesticides that cause decreased birth weight. Thus, there is no scientifically robust data available for evaluating potential mixture effects on this endpoint and for selecting the best model for predicting the mixture effects, i.e. dose-addition (DA) or independent action (IA).

Low birth weight is in both humans and experimental animals a marker for a non-optimal prenatal development and is generally a predictor for increased risk for a long list of diseases later in life, including obesity and type 2 diabetes.

Thus, the project had the following main objectives:

- Investigate whether a mixture of environmentally relevant pesticides cause decreased birth weights at dose levels below NOAELs for the individual pesticides in rats
- Based on existing data submitted for approval of the pesticides, evaluate whether the mixture effect is best predicted by the independent action or the dose-addition model
- Investigate the influences of developmental pesticide exposure on metabolic programming of the offspring, using biomarkers for obesity and type 2 diabetes
- Give input for regulatory considerations

The pesticides included in the mixture were chosen among those approved pesticides in EU that appeared to cause decreased birth weight at doses without signs of maternal toxicity or other toxicity based on the toxicological data in the Draft Assessment Report for each pesticide. Also, the possibility for establishing dose-response relationship and calculate the dose causing 5% decreased birth weight was prioritized. The 6 pesticides chosen were Cyromazine, MCPB, Pirimicarb, Quinoclamine, Thiram, and Ziram. These pesticides were combined in the mixture at doses calculated to cause 5% decreased birth weight.

Two experimental studies in rats were done, i.e. a range-finding study and a large developmental toxicity mixture study (main study), where time-mated Wistar rats were exposed by gavage from gestation day (GD) 7 to pup day (PD) 16. Thus, the offspring were indirectly dosed via the placenta during the prenatal development and via maternal milk after birth.

The range-finding study included 4 groups of 10 time-mated rats and the mixture doses were of 0, 70, 210 and 420 mg/kg/day, i.e. 0%, 12.5%, 37.5% and 75% of the mixture. However, due to toxicity in the highest dose group of 420 mg/kg/day (Mix-75%) during the first 2 days of exposure, the dose level in this group was reduced to 140 mg/kg/day (Mix-25%) from GD 9 and the rest of the dosing period.

In the main study, four groups of 22 were given mixture doses of 0, 28, 90 and 210 mg/kg/day, i.e. 0%, 5%, 16% and 37.5% of the mixture.

The main study as well as the range-finding study showed clearly that doses of the individual pesticides, which were markedly below NOAELs for decreased birth weight, caused decreased birth weight in concert with the other pesticides.

The mixture effect predictions based on data for the single chemicals showed that dose-addition were in good agreement with the observed mixture effect. Also, for the rat dams mixture effects occurred at dose levels where the single substances show no effect on dams, i.e. at the observed NOAELs for maternal toxicity. These results imply that risk assessment based on the NOAEL for one pesticide at a time underestimate the risk for low birth weight and that there is a need for cumulative risk assessment to take account of mixture effects, because of the potentially serious impact of mixed exposure on prenatal development and pregnancy in humans.

It is recommended when considering cumulative risk assessment to include all kinds of chemicals e.g. pesticides, industrial chemicals, environmental contaminants, as substances causing decreased birth weight exist within all of these chemicals classes and humans may be exposed to several of them simultaneously.

In the main study the decreased weight continued into adulthood of the offspring. There was no effect on specific growth rate, food consumption, energy efficiency and oral glucose tolerance. Also, no changes in the investigated organs involved in glucose metabolism (muscle and liver weights) were found and this was further confirmed by histological evaluations of pancreas and liver. At the high mixture dose, PD 16 offspring showed increased weight of fatty tissue relative to the body weight and a tendency to this increase was still seen in males in adulthood. The biomarkers related to metabolic syndrome, obesity and diabetes showed no clear effects on the plasma levels of the measured hormones, except for a significant increase in plasma leptin levels in 5-6 months old female offspring exposed to the highest mixture dose. Some weak trends of dysregulation were discernible for some key regulators of metabolism and adipocyte differentiation in exposed groups, but the results are inconclusive. Overall, no clear signs of metabolic syndrome or obesity were found in the offspring up at the age of 4-5 months. However, this does not rule out that effects could be seen later in life, e.g. in offspring between 1 and 1.5 years old.

Slightly increased nipple retention in male offspring was found in the main study at the highest dose indicating anti-androgenic effect. This taken together with the lack of effects on anogenital distance and reproductive organ weights in the main study, indicate that marked anti-androgenic effects may only be seen at higher doses of the pesticide mixture. The mixture induced slight maternal toxicity at the highest dose in the main study and severe maternal toxicity at an only two times higher dose in the range-finding study. It is therefore most likely not possible to investigate, if there would be marked anti-androgenic effects of this specific pesticide mixture, as the maternal toxicity limits the dose levels that can be studied in rats. However, as there are also indications of anti-androgenic effects for several of the pesticides (MCPB, quinclamine, thiram and MCPB) further studies of these pesticides as well as mixtures based on the potency for anti-androgenic effects appear warranted.

Resume og konklusion

Risikovurdering af pesticider er generelt baseret på No Observed Adverse Effect Levels (NOA-ELs) for et pesticid ad gangen. Mennesker bliver dog typisk udsat for en blanding af flere pesticider samtidigt. Eksperimentelle studier har vist at udsættelse for blandinger af hormonforstyrrende stoffer, inkl. pesticider kan medføre markante kombinationseffekter ved doser, hvor de enkelte stoffer ikke har nogen skadelig effekt.

Nedsat fødselsvægt er fundet for mange pesticider i eksperimentelle undersøgelser, men der er ingen undersøgelser af kombinationseffekter af udsættelse for pesticider, der forårsager nedsat fødselsvægt. Der er derfor ingen robuste data, der kan anvendes til at vurdere potentielle kombinationseffekter og til at vælge den bedste model for at forudsige kombinationseffekter, dvs. dosis-addition (DA) eller Independent Action (IA).

Lav fødselsvægt er hos både mennesker og for forsøgsdyr en markør for ikke-optimal udvikling af fostret og forudsiger generelt en øget risiko for sygdom senere i livet, inkl. fedme og type 2 diabetes.

Projektet havde derfor følgende formål, at:

- Undersøge om en kombination af miljørelevante pesticider forårsager nedsat fødselsvægt ved doser under NOAEL for de enkelte pesticider
- Vurdere om kombinationseffekten forudsiges bedst med DA eller IA-modellen ud fra de eksisterende data indsendt ifm. godkendelse af pesticiderne
- Undersøge om eksponeringen for pesticider under udviklingen påvirker den metaboliske programmering af ungerne ved brug af biomarkører for fedme og type 2 diabetes
- Give input til regulatoriske overvejelser

Pesticiderne blev valgt blandt de godkendte pesticider i EU, der så ud til at føre til nedsat fødselsvægt ved doser der ikke viste tegn på maternal eller anden toksicitet baseret på de toksikologiske data i Draft Assessment Report for hvert pesticid. Muligheden for at etablere dosis-respons sammenhæng og beregne den dosis der giver 5% nedsat fødselsvægt blev prioriteret. De 6 valgte pesticider var Cyromazin, MCPB, Pirimicarb, Quinoclamín, Thiram og Ziram. Disse pesticider blev blandet ift. den dosis, der var beregnet til at medføre 5% nedsat fødselsvægt. Der er i dette projekt udført to studier i rotter, dvs. et pilotforsøg og et stort kombinationsforsøg, hvor tidssparrede Wistar rotter blev doseret med sonde fra gestationsdag (GD) 7 til ungedag (PD) 16. Afkommet blev således indirekte udsat via placenta under den prænatale udvikling og via modermælk efter fødslen.

Pilotforsøget inkluderede 4 grupper med 10 tidssparrede rotter og kombinationsdoserne var 0, 70, 210 and 420 mg/kg/dag, dvs. 0%, 12.5%, 37.5% and 75% af dem i blandingen. På grund af toksiske effekter i den højeste dosisgruppe på 420 mg/kg/dag (Mix-75%) efter 2 dages dosering blev denne dosis nedsat til 140 mg/kg/dag (Mix-25%) fra GD 9.

I det store kombinationsforsøg blev fire grupper af 22 doseret med 0, 28, 90 og 210 mg/kg/dag, dvs. 0%, 5%, 16% and 37.5% af blandingen.

Begge forsøgene viste klart at blandingen af pesticider medførte nedsat fødselsvægt ved doser der var markant lavere end NOAEL for denne effekt for de enkelte pesticider.

De forventede kombinationseffekter beregnet ud fra data for de enkelte pesticider viste at dosis-addition gav en god forudsigelse af den observerede kombinationseffekt. Der var også kombinationseffekter på de drægtige dyr ved doser, hvor de enkelte pesticider ikke påvirkede de drægtige dyr, dvs. under NOAEL for maternel toksicitet. Disse resultater peger på at risikovurdering baseret på NOAEL for et pesticid ad gangen undervurderer risikoen for lav fødselsvægt og at der er brug for kumulativ risikovurdering for at tage højde for kombinationseffekter og de potentielt alvorlige effekter af kombinationseksponering under fosterudvikling og graviditet hos mennesker. Det anbefales at kumulativ risikovurdering inkluderer alle typer af stoffer, f.eks. pesticider, industrikemikalier samt miljøforureninger, da der findes kemiske stoffer, der fører til nedsat fødselsvægt indenfor alle typer af stoffer og mennesker kan være udsat for adskillige af dem samtidigt.

I hovedforsøget fortsatte vægten af ungerne med at være nedsat, da de var blevet voksne. Der var ingen effekter på vækstrate, foderindtag, energieffektivitet og oral glukose tolerance. Der var heller ikke ændringer i de undersøgte organer involveret i glukose metabolismen (muskel- og levervægt) og dette blev yderligere bekræftet ved histologiske undersøgelser af bugspytkirtel og lever. Ved den højeste kombinationsdosis havde ungerne på dag 16 øget vægt af fedtvævet, kropsvægt og en tendens til dette sås også hos hanungerne som voksne. Målinger af biomarkører for metabolisk syndrom, fedme og diabetes viste ingen klare effekter på plasmaniveauer af de målte hormoner, bortset fra et øget plasmaniveau af leptin i 4-5 mdr. gamle hununger udsat for den højeste kombinationsdosis. Nogle svage tegn på dysregulering kunne ses for visse nøgleregulatorer af metabolisme og fedtcelleudvikling, men resultaterne er inkonklusive. Samlet set blev der ikke fundet tydelige tegn på metabolisk syndrom eller fedme hos ungerne op til de var 4-5 måneder. Dette udelukker dog ikke at der kan vise sig effekter senere i livet, f.eks. når ungerne er 1-1,5 år gamle.

Hos hanungerne blev set en svag stigning i antal bibeholdte brystvorter ved den højeste dosis, tydende på anti-androgen effekt. Dette sammenholdt med at der ikke var effekt på hanungernes anogenitalafstand i hovedforsøget og heller ikke på vægte af reproduktionsorganer peger på at klare anti-androgene effekter kun ville kunne ses ved endnu højere doser. Pesticidblandingen medførte svag maternel toksicitet ved den højeste dosis i det store forsøg og alvorlig maternel toksicitet ved en kun to gange højere dosis i pilotforsøget. Det er derfor sandsynligvis ikke muligt at undersøge om der ville være markante anti-androgene effekter af denne pesticidblanding, fordi den materielle toksicitet begrænser, hvor høj dosis kan være. Da der også er indikationen på anti-androgen effekt for flere af pesticiderne (MCPB, quinochlor, thiram and MCPB) ville fremtidige studier af disse pesticider, alene såvel som i en pesticidblanding baseret på potensen for anti-androgen effekt, være relevante.

1. Introduction and background

1.1 Objectives and hypothesis

Risk assessment of pesticides is generally based on the no observed adverse effect levels (NOAELs) for single compounds. However, humans are typically exposed to a mixture of several pesticides. For mixtures of endocrine disrupting chemicals including pesticides, there are experimental evidence showing that substantial mixture effects on reproductive development can occur even though each of the individual chemicals is present at low, non-adverse doses (Hass et al. 2007, Christiansen et al. 2009, Christiansen et al. 2012). We have recently shown that combined developmental exposure of rats to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length in the dams (Hass et al. 2012a,b). These findings have major implications for the human risk assessment, as they imply that the current use of NOAELs for single chemicals may lead to an underestimation of the potential risk for humans exposed to mixtures of chemicals.

Many regulatory bodies have recognized the need for cumulative risk assessment, but the way to group the chemicals and the model for prediction of mixture effects is still under discussion. Two models for prediction of mixture effect are generally considered, i.e. dose-addition (DA) and independent action (IA). A main difference is that the DA model is developed for similarly acting chemicals whereas the IA model is for dissimilarly acting chemicals. However, the term similarly acting can be interpreted in several different ways, ranging from similar molecular mechanism of action over affecting the same toxicity pathway to causing a common effect. Decreased birth weight, which is an indicator of adverse intrauterine environment, is a common effect for many pesticides in experimental studies. There are, however, no data on the effects of combined developmental exposure to pesticides that cause decreased birth weight. Thus, there is no scientifically robust data available for evaluating potential mixture effects on this endpoint and for selecting the best model for predicting the mixture effects, i.e. DA or IA. Decreased birth weight is a developmental toxicity effect that is likely to be induced via many different and in most cases unknown mechanisms of action. In such a case with dissimilarly acting chemicals, the IA model may appear most relevant. However, a recent state of the art on mixtures (combined actions of chemicals in food through dissimilar modes of action) concludes that the DA model should generally be used for chemicals with common effects, irrespective of (often presumed) modes of action (Kortenkamp et al. 2012, EFSA 2013b). Consequently, it is relevant to study combined effects of pesticides with dissimilar modes of action and evaluate the predictive value by applying both the IA and the DA model.

Low birth weight is in both humans and experimental animals a marker for a non-optimal prenatal development and is generally a predictor for increased risk for a long list of diseases later in life, including obesity and type 2 diabetes. A recent Danish study in children born to women working in green houses and thus exposed to mixtures of pesticides has found lower birth weights in exposed children, but increased body fat accumulation from birth to school age (Wolfahrt-Veje et al. 2011). The most widely accepted mechanisms thought to underlie these relationships are those of foetal programming and it is suggested that the foetus adapts physiologically in response to changes in the environment to prepare for postnatal life (de Boe and Harding 2006). Leptin and insulin are recognized biomarkers for risk of obesity and diabetes 2 later in life (Tamashiro and Moran 2010). Thus these markers are considered very relevant for evaluating whether decreased birth weight is related to an altered metabolic programming during development.

Based on the above, the project had the following main objectives:

- Investigate whether a mixture of environmentally relevant pesticides, with dissimilar modes of action, will cause decreased birth weights at dose levels below NOAELs for the individual pesticides, in a developmental toxicity mixture study in rats.
- Based on existing data submitted for approval of the pesticides, evaluate whether the mixture effect is best predicted by the independent action or the dose-addition model.
- Investigate the influences of developmental pesticide exposure on metabolic programming of the offspring, using biomarkers for obesity and type 2 diabetes
- Give input for regulatory considerations on cumulative risk assessment of pesticides causing decreased birth weight, in order to take account of the potentially serious predictive value of this endpoint.

1.2 Background

In the EU Regulation (EC) No 1107/2009 consideration of potential mixture effects is a requirement for human or animal risk assessment of plant protection products (PPP) and residues as it is stated that PPPs “shall have no immediate or delayed harmful effect on human health, including that of vulnerable groups, or animal health, directly or through drinking water (taking into account substances resulting from water treatment), food, feed or air, or consequences in the workplace or through other indirect effects, taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available; or on groundwater” (article 4, paragraph 3(b)) (Kortenkamp et al. 2012).

These more general terms provide the basic legal background for the consideration of potential cumulative risk of PPP ingredients without addressing the necessary next steps. The EU Regulation (EC) No 396/2005 on maximum residue levels specifies in article 36, paragraph 1(c) the necessity to develop a methodology as it is stated “studies and other measures necessary for the preparation and development of legislation and of technical guidelines on pesticide residues, aimed, in particular, at developing and using methods of assessing aggregate, cumulative and synergistic effects”. Based on this the Panel on Plant Protection Products and their residues (PPR) in 2008 developed and published a methodology for the assessment of cumulative and synergistic risks of pesticides to human health which applies to substances with similar mode of action (EFSA, 2008).

A new state of the art science document has proposed a science-based approach for performing cumulative risk assessment also for pesticides with dissimilar modes of action (Kortenkamp et al. 2012, EFSA 2013b). It is recommended to use a tiered framework analysis using DA also for the assessment of mixtures of dissimilarly acting chemicals (Kortenkamp et al. 2012). At lower tiers of the analysis, all chemicals should be assessed, irrespective of their presumed modes of action. At higher tiers, when the risk estimates at lower tiers are considered unacceptable, chemicals known not to contribute to a relevant common adverse outcome can be excluded from the analysis. By way of further refining the analysis, criteria for the grouping of chemicals into common assessment groups based on common adverse endpoint, should be applied.

According to the above recommendations, all substances known to cause reproductive and developmental toxicity should be considered together at lower tiers. However, reproductive toxicity comprises a broad range of different effects, ranging from reductions in fertility to developmental toxicity effects. At higher tiers, such diverse effects will have to be differentiated. For developmental toxicity effects, adverse outcomes are often differentiated into structural abnormalities, functional deficiencies, death of the developing organism and altered growth (ECHA 2009). Altered growth may then be further differentiated into increased or decreased growth, where decreased birth weight constitutes an important endpoint for decreased in utero growth. Decreased birth weight is often seen in experimental studies after exposure to pesticides and is observable from regulatory studies submitted for approval of pesticides. It is a developmental toxicity effect that may be induced via many different and in most cases unknown mechanisms of action. Consequently, independent action (IA) may provide a more accurate prediction of mixture effect on birth weight than dose addition (DA). The state of the art document on mixtures states that there is no current example of a situation in which the concept of IA provides an accurate prediction that is also more conservative (i.e. cautious) than DA, supporting the use of DA as a conservative default in cumulative risk assessment (Kortenkamp et al. 2012, EFSA 2013b). An analysis of the quantitative difference between predictions based on DA or IA suggested that the differences that might be expected in practice are small. The present project aims at investigating whether this also holds true for the endpoint birth weight, following exposure to a mixture of pesticides.

The choice of an appropriate model for the calculation of additivity expectations is essential for assessments of mixture effects, because it is on basis of these additivity expectations that combination effects are judged in terms of synergisms or antagonisms (Hass et al. 2007). Mixture effects according to “no interaction” or “additivity” assumptions can be calculated by using two alternative concepts, dose addition (DA) and independent action (IA). Dose addition looks at mixture effects in terms of a “dilution principle”. It assumes that one chemical can be replaced totally or in part by an equal fraction of an equi-effective dose of another, without diminishing the overall combined effect (Loewe and Muischnek 1926). Dose addition is often used for mixtures composed of chemicals that act through a similar or common mode of action (COT 2002, USEPA 1986; 2000; 2002). Its application to the mixture pesticides in this project could be justified, because all of the pesticides induce a common effect, i.e. decreased birth weight. However, it can be argued with equal justification that the similarity assumption for dose addition is not applicable, because the pesticides most likely produce their effects by many different and in most cases unknown mechanisms of action. For this reason, we also use the alternative concept of independent action to calculate additivity expectations. Independent action assumes that the joint effects of a combination of agents can be calculated by adopting the statistical concept of independent events (Bliss 1939). It is viewed as appropriate for mixtures of chemicals with diverse modes of action (COT 2002), however, dose addition and independent action often yield the same additive mixture effect predictions (Kortenkamp et al. 2012).

One goal for mixture toxicology is to predict the toxicities of mixtures of chemicals when only information on individual components is available (Christiansen et al. 2009). Another issue of relevance to regulatory toxicology concerns the question of mixture effects at low doses. A view that has persisted over the last 30 years is that combination effects do not occur when each chemical is present at doses equal to or lower than their NOAELs (COT 2002). This is thought to be the case with chemicals that act through different mechanisms of action, but not for agents that target similar molecular structures. The NOAEL is the highest tested dose at which no statistically or biologically adverse effects can be identified and is used in regulatory toxicology as a point of departure for establishing e.g. acceptable daily intake (ADI) for pesticides. For the endpoint birth weight in regulatory studies including 20 litters per group, an effect magnitude of 8% will generally turn out being statistically significant. Thus, effects ranging from 0 to <8% reduction in birth weight may appear at NOAEL. If for example 8 pesticides each cause 4% decreased birth weight, the IA model predicts that the mixture effect will constitute a 28% reduction in birth weight. This clearly indicates that adverse mixture effects of dissimilarly acting pesticides would be expected at dose levels below NOAELs for the endpoint birth weight.

The ideal way of designing a mixture study comprises the following sequence of activities:

1. Dose-response studies of the single pesticides using a sufficient number of doses allowing calculation of effects doses (benchmark doses)
2. Predictions of expected mixture effects based on data for the single pesticides and using the appropriate model(s), e.g. DA and/or IA
3. Dose-response mixture study with the pesticides in equi-effective doses, for example a specific bench mark dose
4. Comparison of the expected and observed mixture effects and evaluation of the prediction models.

This is generally an expensive and labour-demanding task and the work-load increases with the number of pesticides to be included in the mixture. However, a low number of test chemicals reduce the possibility for detecting mixture effects and especially for evaluating whether DA or IA is the best prediction model. It is generally a challenge in mixture toxicology to balance these opposite issues in a way that leads to a scientifically sound study design which is also a pragmatic and feasible solution. However, for pesticides, a rather unique possibility for using existing data exists, as pesticides have to be tested using internationally accepted guidelines before approval. For the main endpoint to be examined in this project, i.e. decreased birth weight, relevant background data will be available as birth weight is assessed in the two-generation reproduction toxicity study (OECD TG 416, 2001) and foetal weight is assessed one to two days before birth in the prenatal developmental toxicity study (OECD TG 414, 2001). The number of dose levels in each of these studies is three which may be a limitation when calculating bench mark doses. The prenatal developmental toxicity study, however, often uses higher dose levels than the two-generation study. Thus, for pesticides inducing an effect on both foetal and birth weight there may be more than three relevant effect levels and consequently a better background for calculating bench mark doses. Such pesticides will be selected for the mixture study in this project.

1.3 Prenatal programming

The intrauterine environment supports the development and health of the offspring. Perturbations to this environment can have detrimental effects on the foetus that have persistent pathological consequences later in life (Tamashiro and Moran 2010). Many studies in both humans and animals have provided evidence for the developmental origin of adult disease, i.e. the Barker hypothesis (de Boo and Harding 2006). Low birth weight is in both humans and experimental animals a marker for affected prenatal development and is generally a predictor for increased risk for a long list of diseases later in life, incl. obesity and type 2 diabetes (Cottrell and Ozanne 2007). The most widely accepted mechanisms thought to underlie these relationships are those of foetal programming and it is suggested that the foetus makes physiological adaptations in response to changes in the environment to prepare for postnatal life (de Boo and Harding 2006).

Both leptin and insulin are trophic factors that act during the pre- and postnatal period and can significantly affect development of systems important for energy homeostasis (Tamashiro and Moran 2010). The development of the appetite regulatory system in rodent occurs primarily after birth. A recent study has shown that endogenous leptin is metabolically active in newborn rats (Abdennebi-Najar et al. 2011).

A study in European adolescents has tested the hypothesis that a lower birth weight, as an indicator of an adverse intrauterine environment, is associated with higher leptin levels (Labayen et al. 2011). The results showed a sex-specific programming effect of birth weight on serum leptin levels, i.e. leptin levels were increased only in female adolescents.

The potential effects of intrauterine exposure to pesticides have been studied in children born to women working in green houses in early pregnancy. Birth weights were lower in exposed children, whereas these children showed increased body fat accumulation from birth to school age (Wolfahrt-Veje et al. 2011).

Leptin is one of the cytokines produced in fat tissue. It is secreted in direct proportion to adipose tissue mass and it is one of central regulators of energy homeostasis (Vickers, 2007). We have previously shown that exposure to butylparaben and diisobutylphthalate during development reduced leptin and insulin levels in rat foetuses (Boberg et al. 2008). We have also found that butylparaben stimulate adipogenesis in vitro, increasing lipid accumulation in and the secretions of leptin from mature adipocytes (Taxvig et al., 2012). Overall, these data support the hypothesis that environmental chemicals may contribute to obesity development in humans.

2. Research plan and selection of pesticides

2.1 Research plan

The work has proceeded in several steps and included 5 work packages (WPs 1-5) as well as concurrent activity including Statistics, data analysis and reporting. An overview of the activities and the time schedule is shown in table 1.

Table 1 Overview of project time schedule

| Work Package | Endpoints /activities | 2012 | 2013 | 2014 | 2015 |
|--|--|------|------|-----------|-----------|
| WP 1 Selection of relevant pesticides | Dose-response relationship based on DAR; use in DK | xxxx | | | |
| WP2 Estimating potential mixture effect and selection of dose levels | Calculations of Benchmark doses and predictions based on IA and DA | | xxxx | | |
| WP3 Range-finding study | General toxicity and pregnancy endpoints | | xxxx | xx | |
| WP 4 Developmental toxicity mixture study | General toxicity endpoints in dams Developmental toxicity endpoints | | | xxxxx | xxxxx |
| WP 5 Biomarkers for obesity | Analysis of leptin and insulin; gene expression in fat tissue | | xx | xx | xx |
| Statistics, data analysis and reporting | | | xxxx | xxxxxxxxx | xxxxxxxxx |

2.2 Selection of relevant pesticides (WP1)

The purpose of WP1 was to select 8 relevant pesticides for further evaluations in WP2 and for the experimental mixture studies in WPs 3 and 4.

In a recent evaluation based on the Draft Assessment Report (DAR) we found that among approved pesticides in EU around 175 caused decreased foetal weight or decreased birth weight (EFSA 2012). For a substantial number of those pesticides, signs of maternal toxicity such as decreased maternal body weight gain during pregnancy were seen at the same dose levels. For such pesticides, there may also be combination effects on the dams and this will complicate the evaluation of the developmental toxicity effect. Also, severe dam toxicity may lead to insufficient number of offspring. Therefore, the pesticides for the mixture studies in this project was planned to be chosen among those that appeared to cause decreased birth weight at doses without signs of maternal toxicity, i.e. the 51 pesticides shown in table 2. Among these 51 pesticides, the 8 pesticides were planned to be chosen based on weight of evidence of the information in the DARs using the following general criteria:

1. **Possibility for establishing dose-response relationship and calculation of benchmark doses (BMD).** This means that pesticides for which effects on birth weight are seen at multiple doses will be prioritized over pesticides where an effect has only been found at the highest dose level.
2. **Mode of action.** This means that selection of several pesticides with similar mode of action for causing decreased birth weight was attempted to be avoided as the purpose was to study mixture effects of dissimilarly acting pesticide on birth weight. It was, however, as expected not a criteria of major importance as the mode of action for causing decreased birth weight was unknown in most cases. An exception to this was pesticides affecting the thyroid and/or thyroid hormones and thus causing decreased birth weight. Selection of more than a few of such pesticides was avoided.
3. **Species and strain of experimental animals.** The experimental mixture studies were to be performed using Wistar rats and thus pesticides for which effects on birth weight were seen in Wistar rats were generally prioritized over pesticides showing decreased birth weight in other strains of rats or in mice. We have, however, in an earlier study in our laboratory studied developmental effects in Sprague Dawley (SD) rats and the results indicate that they are very similar to Wistar rats with respect to gestation length, litter sizes and birth weights. Thus, pesticides studied using SD rats were also given a high priority. When a pesticide had been studied only in other strains of rats, we did a case-specific evaluation of the specific strain(s) and based on that decided the priority of the pesticide. Prenatal developmental toxicity studies in rabbits were also available and may provide some information. They were however given lower priority than rat studies, mainly because the mixture studies in the project used rats, but also because it is more difficult to detect maternal toxicity manifested as decreased maternal weight gain in rabbits than in rats. Low priority was also given to pesticides studied only in mice as there may be important especially quantitative differences between rats and mice. It was, however, rarely that there were studies only in mice as most regulatory developmental toxicity and two-generation studies in accordance with the OECD Test Guidelines are performed using rats or rabbits.
4. **Human exposure.** Pesticides with widespread use and human exposure data indicating high likelihood for human exposure were prioritized if more than 8 pesticides were prioritized based on the above criteria.

The data collected in the project EFSA CAG can be found in the database CAPEG (<http://www.efsa.europa.eu/en/supporting/pub/269e.htm>), and was used as a starting point for the evaluation of each pesticide. The selection of the pesticides was based on the data in Draft Assessment Report (DAR) for each pesticide and was done in several steps:

Step 1 Initial selection

- Among the 51 pesticides shown in table 2 was chosen those pesticides where the studies showed effects in rats, i.e. pesticides where the studies showed effect only in rabbits was not chosen.
- Thereafter, the pesticides showing decreased birth weight in the absence of foetal or early pup death was selected
- The results of this initial selection was:
- 7 pesticides without indication of maternal toxicity
- 4 pesticides with some indication for maternal toxicity based on results from other studies
- Among these 11 pesticides, some only caused decreased birth weight at the highest dose.

Table 2. Pesticides causing decreased foetal body weight on gestation day 21 and/or decreased birth weight in the absence of signs of maternal toxicity. All pesticides listed caused *in vivo* effects in studies accepted in the DAR and mainly statistically significant effects are noted. However, in some cases the DAR did not contain sufficient information on statistical analyses, but pesticides leading to effects considered relevant in the DAR are listed ((EFSA 2012).

| | | |
|--------------------------|--------------|-----------------------|
| Acetamiprid | Flumioxazin | Oxamyl |
| Aclonifen | Fluopicolide | Oxasulfuron |
| Amitrole (aminotriazole) | Fosthiazate | Penconazole |
| Bentazone | Imazaquin | Phenmedipham |
| Carbendazim | Ioxynil | Phosmet |
| Chlorpyrifos-methyl | Iprodione | Propamocarb |
| Clopyralid | Isoproturon | Propineb |
| Copper compounds | Isoxaflutole | Prothioconazole |
| Deltamethrin | Maneb | Quinoclamine |
| Desmedipham | MCPA | S-Metolachlor |
| Dichlorprop-P | Mecoprop-P | Tepraloxymid |
| Difenoconazole | Mesotrione | Tetraconazole |
| Dinocap | Metconazole | Thifensulfuron-methyl |
| Esfenvalerate | Metiram | Thiophanate-methyl |
| Ethofumesate | Metrafenone | Thiram |
| Fenprovidin | Metribuzin | Tolylfluanid |
| Fluazinam | Oxadiazon | Ziram |

Step 2 Selection among a broader group of pesticides

As the selection of the 51 pesticides (table 2) as starting point could have excluded some relevant pesticides, a second selection step was undertaken among all of the 175 pesticides causing signs of decreased birth weight. The background for that was that decreased maternal weight gain or food intake during pregnancy can often be listed as maternal toxicity, but may in reality be due to decreased foetal weight. Therefore, some of the excluded pesticides could actually be relevant for inclusion in the mixture study.

- The results of this lead to a total 51 pesticides (the 11 from step 1 and 40 more from step 2) which were identified to show effects on birth weight in the absence of foetal or early pup death
- Among these 51 pesticides, those not expected to cause problematic maternal toxicity effects at relevant dose levels were selected. The background for this selection was data from reproductive toxicity studies as well as 28- or 90-days repeated dose toxicity studies
- Also, pesticides showing dose-response relationship with effect at more than one dose were selected to improve the possibility for estimation BMDs.
- 10 pesticides were selected, i.e. Cyromazine, Fluazinam, Fluopicolide, MCPB, Pirimicarb, Quinoclamine, Thiabendazole, Thiram, Triasulfuron, and Ziram.

Step 3 Final selection

The final selection was based on the toxicological data for the pesticides. Fluazinam was excluded due to risk of other toxicity than decreased birth weight. Some studies have shown liver effects at far lower doses than the bench mark dose 5 (BMD5) for decreased birth weight, and as some other of the selected compounds have shown also indications for weak liver toxicity, although at high doses, nevertheless their combined liver toxicity might interfere with our test dose ranges.

Thiabendazole was excluded as the dose response data was judged as insufficient for the dose-response analysis: effects required for the calculation of the combined effects would have to be estimated mainly from extrapolations, a scenario which we considered as too uncertain. Although the Advisory Group discussed omitting either Ziram or Thiram due to their chemical similarities, these were kept on the final list as these appeared to be better candidates than the two that were excluded.

The final selection of the 8 pesticides based on the toxicological data was therefore: Cyromazine, Fluopicolide, MCPB, Pirimicarb, Quinoclamine, Thiram, Triasulfuron, and Ziram.

2.3 Estimation of potential mixture effect and selection of dose levels (WP2)

The NOAELs or LOAELs identified in the DARs for the 8 selected pesticides were not likely to be readily suited as input data for modelling the predicted mixture effect, because NOAELs and LOAELs do not describe effect magnitudes. Depending on the power of the study, the effects potentially associated with NOAELs may be 5%, 10% or 20% effect. For the endpoint birth weight in regulatory studies including 20 litters per group, an effect magnitude of 8% will usually be statistically significant. Thus, effect ranging from 0 to below 8% may be present at the NOAEL. Therefore, before initiating the experimental studies, calculations of bench mark doses (BMDs) for each of the 8 selected pesticides were performed. The predicted mixture effect was then estimated using the two models IA and DA.

Dose-response modelling and bench mark dose estimation

Bench mark doses (BMDs) for each of the 8 selected pesticides for effects on birth weight were determined by the expert statistician. A 5% reduction compared to control birth weight was defined as appropriate benchmark, and the estimation of the BMD5 was based on all available data sources that were judged as comparable to our lab conditions. This low effect value was chosen as it will be most likely above the statistical detection limit for the planned experimental design, and therefore detectable as significant under controlled false-positive ($\alpha=5\%$) and false negative error rates ($\beta=20\%$). For some compounds data were available from more than one study, and to achieve a meaningful pooling the reported absolute weight values data were normalized by their control mean to a relative effect scale. Only means were used in data analysis, and if results were reported for both genders, an overall mean from both were used. Nonlinear regression analyses were performed using the best-fit approach (Scholze et al. 2001), i.e. a variety of nonlinear regression functions were fitted independently to the same data set and the best-fitting model was selected using a statistical goodness-of-fit criterion.

Dose-response data and regression curve estimates are shown for the 8 compounds in Figure 1, where the dots are mean estimates and coloured according to their study origins. Vertical lines correspond to the control and 5% reduction level, the horizontal line indicates the dose that will most likely produce a 5% reduction in birth weight (BMD5).

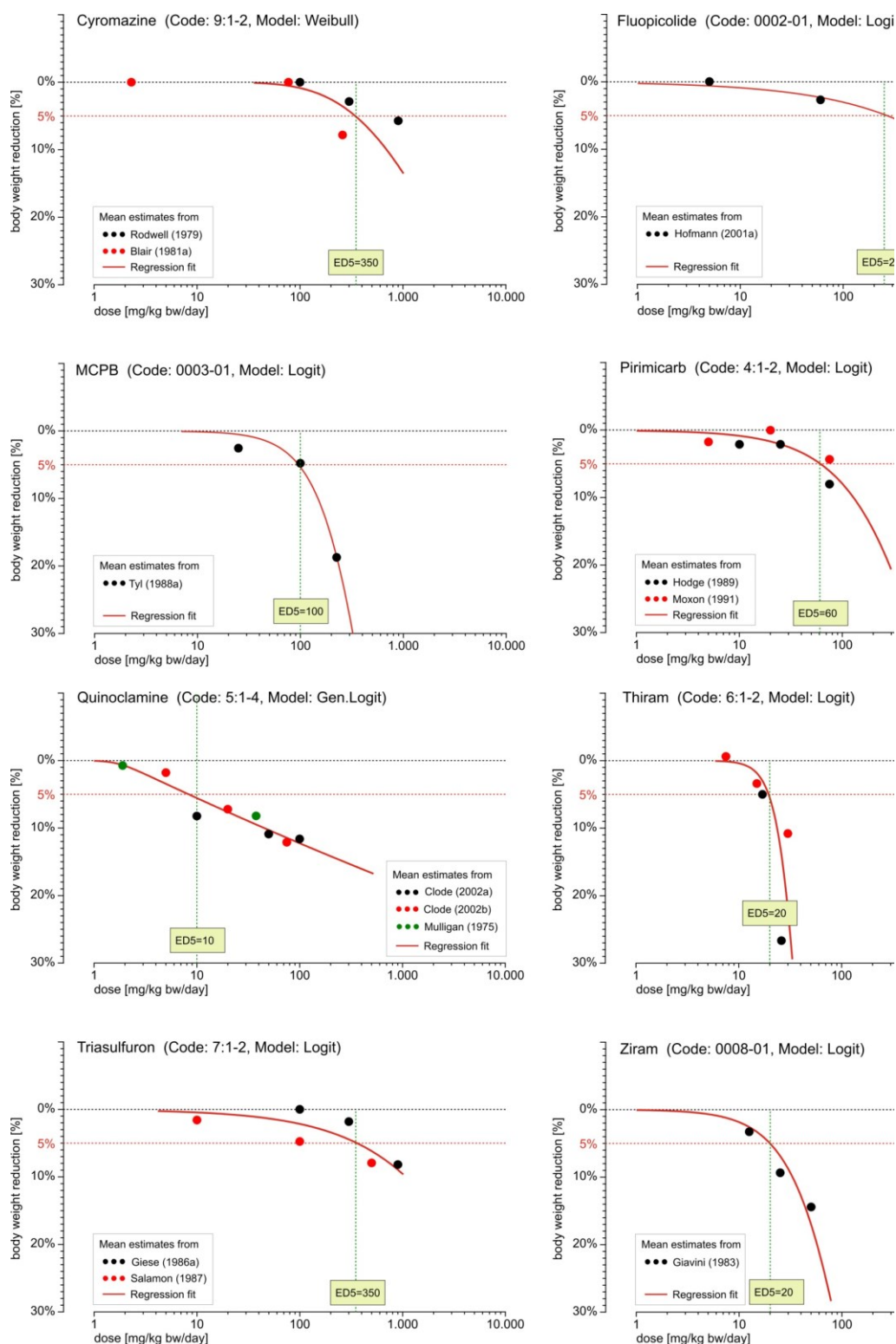


Figure 1. Dose-response data and regression curve estimates for the 8 pesticides. The dots are mean estimates and coloured according to their study origins. Vertical lines correspond to the control and 5% reduction level, the horizontal line indicates the dose that will most likely produce a 5% reduction in birth weight (ED50).

Table 3. Comparison of BMD 5 and toxicities described for reproductive and repeated dose studies. All doses are mg/kg bw/day. Notable effects near BMD5 are underlined.

| | Maternal effects | Other toxicities | BMD 5 (rounded) |
|---------------------|---|---|--------------------|
| Cyromazine | <u>Maternal toxicity LOAEL 300</u> or 600 mg/kg, reduced maternal bw (no data on statistics). Clinical signs at 600 mg/kg (increased activity, red nasal discharge, clear oral discharge and inactivity, no details about number of dams affected). | Prenatal death in rabbits, not in rats. 28-day study in rats: <u>anaemia at 232 mg/kg</u> . Acute toxicity study at high doses, no NOAEL determined. | 344 (350) |
| Fluopicolide | <u>Maternal toxicity LOAEL 700</u> or <u>50 mg/kg</u> (decreased bw gain F0 during gestation (increased mean absolute and relative kidney and liver weights and reduced spleen weights in F0 males and females at 200)). | Rabbit prenatal tox.: premature delivery. 28-day study: <u>liver hypertrophy at 100 and 179 mg/kg</u> . Acute toxicity studies performed at high doses only; no NOAEL determined. | 261 (250) |
| MCPB | <u>Maternal toxicity LOAEL 100 mg/kg</u> , reduced bw and bw gain, transiently reduced food intake at all dose levels. Alopecia at high dose only (no details about number of affected dams). | | 96 (100) |
| Pirimicarb | <u>Maternal toxicity LOAEL 75 mg/kg</u> , decreased bw gain and food consumption. | Study from open literature: Rats dosed GD 18: reductions in both adult and foetal brain acetyl cholinesterase activity at 2 mg/kg. Tremors, salivation, meiosis, dyspnea and piloerection. Neurological effects not noted at higher doses in DAR teratology studies. Acute toxicity study showed signs of <u>systemic toxicity at all dose levels from 100 mg/kg</u> (no data on specific effects at 100 mg/kg listed). | 61 (60) |
| Quinoclamine | <u>Maternal toxicity LOAEL 20 or 75 mg/kg</u> . | 28 and 90-day studies: <u>liver effects from 15-50 mg/kg</u> . Acute tox. LOAEL 200 mg/kg. | 8 (10) |
| Thiram | <u>Maternal toxicity LOAEL 17 or 3 mg/kg</u> , decreased food consumption and bw, mainly during gestation. | 90-day studies: Neurological effects at high doses (150 mg/kg). Acute toxicity studies performed above 500 mg/kg; no NOAEL. | 19 (20) |
| Triasulfuron | <u>Maternal toxicity LOAEL 250 or 300 mg/kg</u> , decreased bw gain and bw. | 28-day study: <u>anaemia at 228 mg/kg</u> , kidney and bladder effect at 686 mg/kg. Acute toxicity studies performed at high doses only, no NOAEL determined. | 359 (350) |
| Ziram | <u>Maternal toxicity LOAEL 12.5 or 25 mg/kg</u> , reduced maternal bw gain. Two other prenatal studies show reduced foetal bw at doses from 20 and 64, respectively. No data are presented in DAR from those studies. Dam effects on food intake and bw at same doses, hair loss (not specified). | 90-day study: <u>anaemia at 21 mg/kg</u> . 28- and 90-day studies: <u>liver degeneration at 15 and 12 mg/kg</u> . Thyroid effects mainly in chronic studies. Acute toxicity studies performed at doses above 250 mg/kg; no NOAEL determined. | 20 (20) |

Toxicity considerations

Considerations on maternal toxicity and other toxicities were included in the compound selection so that compounds were not included if maternal toxicity was seen at same dose ranges as birth weight reductions. Several pesticides induced decreased maternal body weight gain and food intake, but this was not considered as exclusion criterion, as these observations may be associated to the reduced weight of the foetuses. This might explain why most of the selected compounds showed indications for reduced maternal body weight gain at dose ranges affecting pup birth weight. More severe types of maternal toxicity have been reported at higher doses for most of these compounds.

For the eight selected compounds further evaluations were performed after the estimation of BMD5 levels, as BMD5s were compared to dose levels showing maternal toxicity or other toxicity in reproductive studies or other repeated dose studies. This comparison is presented in Table 3.

The comparisons of BMD5s for birth weight reduction to dose ranges showing maternal toxicity or other toxicity revealed the following:

- For all compounds (except Quinoclamine) the LO(A)ELs for maternal toxicity were close to BMD5 for birth weight reduction. It should be noted that these LO(A)ELs were based on reduced maternal body weight gain and food intake during gestation, and thus may be directly related to the lower weight of foetuses in the uterus. As discussed at the Advisory Group meeting in February and as mentioned earlier, this is not considered as true maternal toxicity.
- For some compounds other toxicities were reported at dose levels close to BMD5s
 - Liver effects (hypertrophy) were reported for Fluopicolide, Quinoclamine and Ziram.
 - Anaemia was reported for Cyromazine, Triasulfuron and Ziram.
 - For Pirimicarb unspecified systemic toxicities were reported in one study at a dose just above the BMD5, but no systemic toxicities were reported in reproductive toxicity studies at the same or lower dose levels.

No estimates of mixture effects on maternal toxicity or other toxicities were modelled, as data were too scarce and unsuited for reliable dose-response modelling. However, these findings provide clear indications that the combination of these compounds at their BMD5s could lead to effects beyond a reduction in birth weight. Especially risk of liver effects, anaemia and reduced maternal weight gain could be ruled out.

These expectations of toxicity at BMD5 were considered in the selection of mixture doses

Mixture ratio and mixture dose selection

The mixture ratio for the in vivo studies was based on BMD5 for each compound. Subfractions of the mixture of compounds (each at their BMD5) were used for the experimental studies.

The aim of the pilot study was to identify dose levels at which the mixture reduced birth weights without causing toxicity in the dams. As noted above however, some reductions in dam body weight gain were likely to be observed at the same dose levels as those causing reduced birth weight of pups.

Table 4 describes the predicted decrease in birth weight when including all 8 pesticides in the mixture at fractions of the BMD5-mixture. Fractions of 12.5%, 40% and 75% were suggested as relevant mixture doses for the range-finding study based on the predicted decreases in birth weight.

Table 4. Predicted decrease in birth weight based on dose-addition, independent action and no mixture effect for mixture of 8 pesticides.

| Group | Mixture dose (mg/kg bw/day) | Dose addition | Independent action | No mixture ef- fect |
|------------|--------------------------------|---------------|-----------------------|------------------------|
| Control | 0 | 0% | 0% | 0% |
| 12,5% BMD5 | 171 | 5% | 7% | 1.6% |
| 40% BMD5 | 548 | 14% | 15% | 3.1% |
| 75% BMD5 | 1028 | >30% | 28% | 4.5% |

It may be noted that no calculations are presented for 100% of the BMD5-mixture, as this dose was likely cause toxicity in dams. Importantly, table 4 shows that at 75% of the BMD5-mixture, a 4.5% reduction in birth weight is predicted even in the absence of mixture effects, i.e. it corresponds to the most potent compounds at that mixture dose. A 4.5% reduction in birth weight may be detectable as a statistically significant response in the pilot study. Therefore, it was not considered relevant to go to even higher mixture doses in the range-finding study.

It was possible that maternal toxicity could occur in the pilot study at the highest dose level and possibly the middle dose level, if mixture effects on e.g. liver toxicity, anaemia or maternal toxicity occurred. However, to fulfil the aim of the range-finding study it was considered necessary to investigate the possibility that no mixture effects occur. Therefore, it was necessary to include the dose of 75% of BMD5 at which a slight birth weight reduction is expected even if no mixture effects occur. This dose implies the risk of marked maternal toxicity and loss of dams if mixture effects of all pesticides occur for these endpoints.

The reductions in maternal weight gain seen in other studies at doses near BMD5 indicate that each compound may contribute to a mixture effect on maternal weight gain. If dose addition occurs for the endpoint maternal body weight gain, then 1/8 (12.5%) of the BMD5-mixture is likely to cause reduced maternal weight gain during pregnancy.

Interestingly, the data in Table 4 show that if mixture effects on birth weights do occur, then the models dose addition and independent action predict fairly similar responses for each of the presented dose levels.

Final selection of pesticides and mixture dose levels

Unexpectedly, Fluopicolide and Triasulfuron turned out to be impossible to obtain from our suppliers and these therefore had to be omitted and the studies were performed with a mixture of 6 pesticides. The mixture predictions when including 6 pesticides in the mixture are shown in Table 5. The predicted effect sizes using the 6 compound mixture reveals largely the same predicted effect sizes as for the 8 compound mixture. At the highest dose group there appears to be a larger difference between the dose addition model and the independent action model than for the mixture of 8 pesticides.

Table 5. Predicted decrease in birth weight based on dose-addition, independent action and no mixture effect for mixture of 6 pesticides, i.e. Cyromazine, MCPB, Pirimicarb, Quinoclamine, Thiram, and Ziram.

| Group | Mixture dose (mg/kg bw/day) | Dose addition | Independent action | No mixture ef- fect |
|------------|--------------------------------|---------------|-----------------------|------------------------|
| Control | 0 | 0% | 0% | 0% |
| 12.5% BMD5 | 70 | 4% | 4% | 1.6% |
| 37.5% BMD5 | 210 | 11% | 10% | 2.9% |
| 75% BMD5 | 420 | 28% | 18% | 4.3% |

3. Experimental mixture studies in rats

3.1 Introduction

Two experimental studies in rats were done, i.e. a range-finding study in WP3 and a main study in WP4. An overview of the studies is shown in Table 6 and a more detailed description is given below.

Most endpoints in the studies were similar to those included in OECD guidelines for reproductive and developmental toxicity, while others were included due to their sensitivity for detecting effects on prenatal programming. In addition to the endpoint birth weight, other endpoints were included covering effects on male and female offspring during the postnatal development of the pups, up to and including sexual maturation. An important reason for including endpoints other than the main endpoints, i. e. birth weight and prenatal programming, was to have a sufficient background for evaluating the sensitivity and thus the relevance of the effects on birth weight and prenatal programming. Thus, some endpoints for anti-androgenic effects (anogenital distance and nipple retention) were included. Other endpoints considered important, such as weight and histopathology of target organs were also to the extent possible included in the main study. Toxicity to the dams were also assessed and evaluated in relation to the developmental toxicity effects.

Concerning prenatal programming and risk for obesity, the growth of the animals were assessed more often than normally from birth to young adulthood. Food consumption was assessed to investigate potential effects on appetite. Blood samples were taken shortly after birth and after sexual maturation around the age of 4-5 months and analysed for obesity related hormones and pro-inflammatory cytokines. Fat tissues were sampled after sectioning of the offspring and analysed for gene expression changes e.g. in PPAR γ that is known to be involved in obesity development.

Table 6 Overview of the range-finding and the mixture study, study design and effects

| Study design | Effects in adults/dams | Effects in offspring, pup day 1-24 | Effects in offspring after pup day 24 to around 3 months |
|---|---|---|--|
| Range-finding, pregnant animals, control and 3 doses, 10 time-mated female rats per group | Body weight gain during pregnancy and lactation, gestation length, litter size | Birth weight, growth and survival, AGD, nipple retention, biomarkers for obesity | Not included |
| Developmental toxicity mixture study, pregnant animals, control, 3 doses, 22 time-mated female rats per group | Body weight gain during pregnancy and lactation, gestation length, litter size, hormones. | Birth weight, growth and survival, AGD and nipple retention, biomarkers for obesity | Body weight gain, food consumption, sexual maturation, biomarkers for obesity, weight and histopathology of target organs, i.e. liver, pancreas. |

The purpose of WP 5 was to examine biomarkers for obesity in both the dams and in the offspring to find out whether there are indications of effects related to increased risk for obesity or type-2 diabetes. Blood samples were taken shortly after birth and after sexual maturation around the age of 4-5 months and analysed for obesity related hormones and pro-inflammatory cytokines, such as leptin, insulin, IL-1 beta, MCP-1, IL-6, TNF-alpha and PAI1 active. The number of samples from the range-finding study and from the developmental toxicity mixture study is shown in Tables 4 and 5, respectively. Fat tissues were sampled after sectioning of the offspring and analysed for gene expression changes e.g. in PPAR γ that is known to be involved in obesity development. The protein biomarkers for early obesity parameters were analysed using Luminex 100 S platform and kits from e.g. Millipore. The results obtained in this WP were evaluated together with the results from WP 3 and WP4, especially the endpoints maternal body weight gain during pregnancy and lactation, birth weight and pup body weight gain during the lactation period, the period of sexual maturation and young adulthood and the time for sexual maturation.

3.2 Range-finding study in pregnant animals

3.2.1 Introduction

The purpose of the range-finding study was to obtain data allowing selection of relevant dose levels for the large developmental toxicity mixture study in WP4. Thus, a small-scale range-finding mixture study in time-mated Wistar rats was performed in order to minimize potential animal suffering. The pesticides were combined in the mixture based on the estimated BMD5 of each individual pesticide on birth weight i.e. at doses causing a 5% decreased birth weight. This mixture was studied at doses lower than this effect dose. Four groups of 10 time-mated animals were exposed during pregnancy and lactation, where the study ended. Table 7 shows the effects assessed, the specific endpoints, the time/age for the recording of the endpoints and number of samples.

Table 7 Effects assessed the range-finding study, specific endpoints, time/age for the recording of the endpoints and number of samples

| Effect | Endpoints and time/age | No. of samples |
|------------------------|---|--|
| Maternal toxicity | Body weight during pregnancy and lactation; GD 7-21 and PD 1-16 | 10 dams per group during pregnancy and 6-9 dams per group during lactation |
| | Gestation length; GD 22-24 | Biomarkers: blood samples from 6-9 dams per group |
| | Litter size; GD 22-24 | |
| | Implantation scars; PD 22 | |
| Developmental toxicity | Biomarkers for obesity; PD 22 | |
| | Birth weight; PD 1 | 6-9 litters per group and ~10 pups per litter = |
| | Pup growth and survival; PD 1, 6, 14, 22 | 60-90 pups per group |
| | AGD; PD 1 | Biomarkers: blood samples from ~1 male and 1 female per litter, i.e. 12-18 blood samples per group |
| | Nipple retention; PD 14 | |
| | Biomarkers for obesity; PD 16 | |

GD = Gestation day; PD = pup day

The specific questions addressed were:

- Do the mixture dose levels cause marked maternal toxicity or marked effects on pregnancy parameters, such as litter size and pup survival? If so, the doses need to be lowered in the main study, because such effects would be incompatible with animal welfare considerations and with the purpose of the main study.
- Does the pesticide exposure seem to cause mixture effects on birth weight? As the range-finding study used much fewer litters than the main study, indications of this were regarded as sufficient for showing the relevance of the main study.
- Does the mixture exposure seem to affect biomarkers for obesity? As the range-finding study used much fewer litters than the main study and samples from the perinatally exposed offspring were taken only in young pups, indications of effects on obesity biomarkers would be regarded as interesting, but would not be needed for showing the relevance of assessing these parameters in both pups and adult offspring in the main study.
- Does the mixture exposure induce other relevant developmental toxicity effects, incl. effects on sexual development? If so, these results are relevant when considering the endpoints to be included in the main study.

The results of this study formed the basis for planning both dose levels and additional endpoints to be included in the main study.

3.2.2 Materials and methods

Animals and dosing

Animal experiments were carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use. Four groups of 10 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of the mixture of the 6 pesticides from gestation day (GD) 7 to pup day (PD) 16. Thus, the offspring were indirectly dosed via the placenta during the prenatal development and via maternal milk after birth.

The vehicle used were corn oil (product no. C8267-2.5L from Sigma/Aldrich). The test compounds were Cyromazine (97%) CAS: 66215-27-8 (product no.:551295-25G from Sigma/Aldrich), MCPB (99.8%) CAS: 94-81-5 (product no: 36145-20G from Sigma/Fluka), Pirimicarb (98.7%) CAS: 23103-98-2 (product no: 45627-15G from Sigma/Fluka), Qinoclamine (99.9%) CAS: 2797-51-5 (product no: 32719-3G from Sigma/Fluka), Thiram (99.9%) CAS:137-26-8 (product no 45689-5G from Sigma/Fluka), Ziram (98.2%) CAS:137-30-4 (product no: 45708-5G from Sigma/Fluka).

The same batch of substances was used in both studies. The mixture ratio was based on the estimated BMDs for 5% decreased birth weight for the individual pesticides. The mixture doses were of 0, 70, 210 and 420 mg/kg/day, i.e. 0%, 12.5%, 37.5% and 75% of this mixture. However, due to toxicity in the highest dose group of 420 mg/kg/day (Mix-75%) during the first 2 days of exposure, the dose level in this group was reduced to 140 mg/kg/day (Mix-25%) from GD 9.

Consequently, Mix-75% was only used on GD7 and 8, and from GD9 and throughout the rest of the study all animals in this group were dosed with Mix-25%. Thereby the Mix-75% dose group was no longer the highest dose group in the study as the Mix-37.5% dose group was now the highest. The doses of the individual pesticides and the sum of them, i.e. the mixture doses, are shown in table 8. The names used for the four groups throughout this report will be: control, Mix-12.5%, Mix-25% (or Mix-75% on GD 7-8) and Mix-37.5%.

Table 8 Composition of pesticide mixture used for the range-finding study in pregnant female animals (doses in mg/kg bw/day)

| Pesticide | Control | Mix-12.5% | Mix-25%** | Mix-37.5% | 75% BMD5* |
|-------------------|---------|-----------|-----------|-----------|-----------|
| Cyromazine | 0 | 43.75 | 87.5 | 131.25 | 262.5 |
| MCPB | 0 | 12.50 | 25.0 | 37.50 | 75.0 |
| Pirimicarb | 0 | 7.50 | 15.0 | 22.50 | 45.0 |
| Quinoclamine | 0 | 1.25 | 2.5 | 3.75 | 7.5 |
| Thiram | 0 | 2.50 | 5.0 | 7.50 | 15.0 |
| Ziram | 0 | 2.50 | 5.0 | 7.50 | 15.0 |
| Pesticide mixture | 0 | 70 | 140 | 210 | 420 |

* Only used in the study on GD 7-8. Hereafter changed to Mix-25% due to maternal toxicity

** From GD 9 to PD 16

The substances used were corn oil (vehicle) (Sigma-Aldrich, Brøndby, Denmark), and cyromazine, MCPB, pirimicarb, quinoclamine, thiram and ziram. All pesticides were purchased in a technical quality from VWR- Bie & Berntsen, Herlev, Denmark.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Techniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lyngø, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). The cages were situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lux, temperature $21 \pm 2^\circ\text{C}$, humidity $50\% \pm 5\%$, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided ad libitum.

Pregnancy and lactation

The animals were observed twice daily for signs of toxicity and dam body weights were recorded daily. The expected day of delivery, GD 23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as most of the animals gave birth on GD 23.

The weights of dams and individual pups were recorded after delivery and the pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The endpoints also included gestation length, number of implantation scars in the uterus of the dam PD 16, body weights of pups at PDs 6 and 14, anogenital distances in the new-born pups and nipple retention PD14.

Caesarean sections were performed for two animals, one from the control group and one from the group dosed with 210 mg/kg/day (37.5%), because these animals showed signs of early birth on GD 21. The endpoints recorded included number of implantations, number of dead and live foetuses, sex ratio in the litters, and foetal body weights for the live foetuses.

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in the offspring at the registration of birth using an ocular stereomicroscope. On PD 14, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring.

Section on PD 16

The offspring were weighed and decapitated. Blood samples were collected in 4 ml vacutainers with sodium heparin for analysis of biomarkers for obesity and analysis of glucose level, pooled in one sample for males and one sample for females within each litter.

Dams were decapitated in CO₂/O₂ anaesthesia and the numbers of uterine implantations were counted. The liver was examined macroscopically and the weight was recorded. Blood samples were collected in 10 ml vacuettes with sodium heparin for analysis of biomarkers for obesity. All blood samples were stored on ice until centrifugation within one hour. The samples were centrifuged at 4000 rpm, 4 degrees for 10 minutes and the supernatant were transferred to 2 CM tubes with 160µl in one of them and the rest in the other and frozen at -80 degrees.

Metabolic Biomarkers

Plasma levels of a selection of metabolic hormones were measured using LUMINEX technology and the MILLIPLEX® MAP Rat Metabolic Bead panel kit (#RMHMAG-84K) from Millipore, (Billerica, MA, USA). This kit was used for the simultaneous quantification of multiple analytes including: Amylin (active), C-peptide, GLP-1, Glucagon, Insulin, Leptin, and TNFα. The measurements were performed on plasma samples from dams as well as 16 day old male and female offspring. All analyses were performed according to the manufacturer's protocol, applying a Luminex IS 100® (Luminex Corporation, Austin, TX, USA). Acquired fluorescence data were analysed using the Bioplex ver. 5.0 software (Bio-rad Laboratories; USA). Plasma glucose concentrations were determined on an ABX Pentra 400 (Horiba ABX, Montpellier, France) using a commercially available kit (ABX Pentra Glucose HK CP cat.no. A11A01667, Montpellier, France).

Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were analysed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. Birth weights were analysed using the number of offspring per litter as covariate and AGD and organ weights were analysed using body weight as a covariate. Additionally, the mean birth weights in the litters were analysed using Fisher's Exact Test. The presence or not of nipples/areolas in male offspring within each litter was analysed using Fisher's Exact Test and the litter as the unit. Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$. All analyses were performed using SAS Enterprise Guide 4.3, SAS Institute Inc., Cary, NC, USA.

Statistical analysis of metabolic biomarker data was performed using Sigma Plot v.11.0 (Systat Software Inc.). Data were tested for normality and homogeneity of variance. Data were analysed by one-way analyses of variance (ANOVA) and, if significant followed by Dunnett's post hoc test. Significance was judged at $p < 0.05$.

3.2.3 Results

Effects during gestation

After two days of dosing, i.e. after recording of the body weights, but before the dosing on GD 9, symptoms of toxic effects (impaired breathing) and weight loss were seen especially in the highest dose group of Mix-75%, but to some extent also at Mix-37.5% (Figure 2). Consequently, the Mix-75% dose was decreased to Mix-25% from GD 9.

On GD 10, one dam from Mix-75/25% (M32) still showed signs of impaired breathing before dosing and was not dosed on that day. The animal was dosed again on GD 11 with Mix-25%, but since the symptoms of impaired breathing reappeared, the dosing of this animal was stopped on GD 12 and data from this litter was excluded from further analysis. One dam from Mix-37.5% (M22) showed similar symptoms from GD 18 and this animal was not dosed during the remainder of the study and data from this litter was excluded from the results.

Due to signs of early parturition on GD 21 (rat dams give birth on GD22-23), caesarean sections were performed on two animals, one from the control group and one from the group dosed with Mix-37.5%. As this occurred in one control dam and in only one exposed dam, these findings are not considered to be related to the exposure.

The body weight gain from GD 7 to GD 21 in the animals giving birth was similar to control values in the group dosed with Mix-12.5%, but appeared to be decreased in the groups dosed with Mix-25% and Mix-37.5%, however these differences were not statistically significant (Table 9).

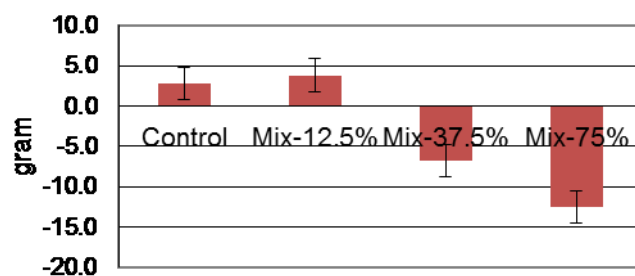


Figure 2. Maternal weight gain GD 7-9 in all time-mated animals in the range-finding study. Results shown are mean±STD, N= 10

Table 9 Pregnancy and litter data in the range-finding studyData represent group means, based on dams or litter means \pm STD

| | Control | 70 mg/kg Mix-12.5% | 140 mg/kg Mix-25% ^a | 210 mg/kg Mix-37.5% |
|--|---------------------|-----------------------|-----------------------------------|------------------------|
| Time-mated females (no.) | 10 | 10 | 10 | 10 |
| Toxicity symptoms after GD 9 (no.) | 0 | 0 | 1 | 1 |
| Non-pregnant (no.) | 1 | 2 | 0 | 2 (3 ^b) |
| Caesarean section GD 21 (no.) | 1 | 0 | 0 | 1 |
| Pregnant, but no pups on PD1 (no.) | 0 | 1 | 0 | 0 |
| Females giving birth/litters (no.) | 8 (7 ^c) | 7 | 9 | 6 |
| Maternal bw gain GD7-GD21 (g) | 71.6 \pm 15.5 | 78.0 \pm 11.9 | 62.0 \pm 12.4 | 58.0 \pm 12.0 |
| Maternal bw gain GD7- PD1 (g) | 12.1 \pm 10.6 | 10.0 \pm 6.2 | 2.1 \pm 6.6 | -4.7 \pm 10.3* |
| Gestational length (d) | 23.1 \pm 0.4 | 22.9 \pm 0.4 | 22.9 \pm 0.4 | 23.0 \pm 0.0 |
| Litter size, live pups (no.) | 9.4 \pm 4.0 | 11.7 \pm 2.3 | 9.9 \pm 3.8 | 11.3 \pm 1.4 |
| Foetal and pup mortality, GD7-PD16 (%) | 1.3 \pm 3.4 | 1.1 \pm 2.9 | 3.6 \pm 7.9 | 0.0 \pm 0.0 |
| Birth weight, male pups (g) | 6.3 \pm 0.4 | 6.1 \pm 0.7 | 5.9 \pm 0.8 | 5.9 \pm 0.2 |
| Birth weight, female pups (g) | 6.0 \pm 0.3 | 5.6 \pm 0.5 | 5.6 \pm 0.8 | 5.6 \pm 0.1 |
| AGD, males (units) | 24.4 \pm 0.9 | 22.5 \pm 0.7* | 21.7 \pm 0.4* | 22.1 \pm 0.6* |
| AGD, females (units) | 13.5 \pm 0.7 | 12.0 \pm 0.6* | 11.5 \pm 0.5* | 12.0 \pm 0.7* |
| Body weight, PD 6, male pups (g) | 12.3 \pm 1.8 | 12.1 \pm 1.8 | 12.2 \pm 2.3 | 11.5 \pm 0.8 |
| Body weight, PD 6, female pups (g) | 11.9 \pm 1.7 | 11.4 \pm 1.1 | 11.8 \pm 2.2 | 11.2 \pm 0.8 |
| Body weight, PD 14, male pups (g) | 25.4 \pm 4.9 | 25.0 \pm 4.4 | 26.5 \pm 6.5 | 23.9 \pm 1.7 |
| Body weight, PD 14, female pups (g) | 25.0 \pm 5.0 | 24.1 \pm 3.4 | 26.2 \pm 6.0 | 23.5 \pm 1.7 |
| Nipple retention, PD 14, males, no. | 0.02 \pm 0.05 | 0.00 \pm 0.00 | 0.02 \pm 0.05 | 0.22 \pm 0.39 |
| Nipple retention, PD 14, females, no. | 12.3 \pm 0.3 | 12.3 \pm 0.3 | 12.1 \pm 0.8 | \pm 0.3 |

* p < 0.05

- 1) Exposed to Mix-75% from GD 7-8 and Mix-25% from GD 9-PD16
- 2) The dosing of one of the non-pregnant females was already stopped due to toxicity
- 3) One litter with only 1 male pup was terminated on PD 6 due to very low pup weight. It is common that litters with only 1-2 pups leads to insufficient maternal care, most likely due to insufficient stimulation of milk production in the dam

Pregnancy data (Table 9)

The maternal body weight gain from GD7 to PD 1 appeared decreased in the groups exposed to Mix-25% and Mix-37.5%, but the difference was only statistically significant in the Mix-37.5% group ($p=0.003$). Gestational length, number of live born pups per litter and the mortality of foetuses and pups were unaffected by the exposure.

Birth weight and pup weight (Table 9, Figure 3)

The birth weight of the pups seemed dose-dependently decreased compared to control at all doses. In the Mix-37.5% group, the birth weight was 7-8% lower than control values, and the decreases were 4% and 5% in the groups exposed to Mix-12.5% and Mix-25%, respectively. However, the differences were not statistically significant in any of the dose groups. The number of litters with mean birth weight below 6 grams was also dose-dependently increased and the difference was statistically significant from controls in the group exposed to the highest dose of Mix-37.5%.

Pup body weights on PDs 6 and 14 were similar to control values in the groups exposed to Mix-12.5% and Mix-25%, but appeared 6-7% lower in the group exposed to Mix-37.5%. However, the difference was not statistically significant.

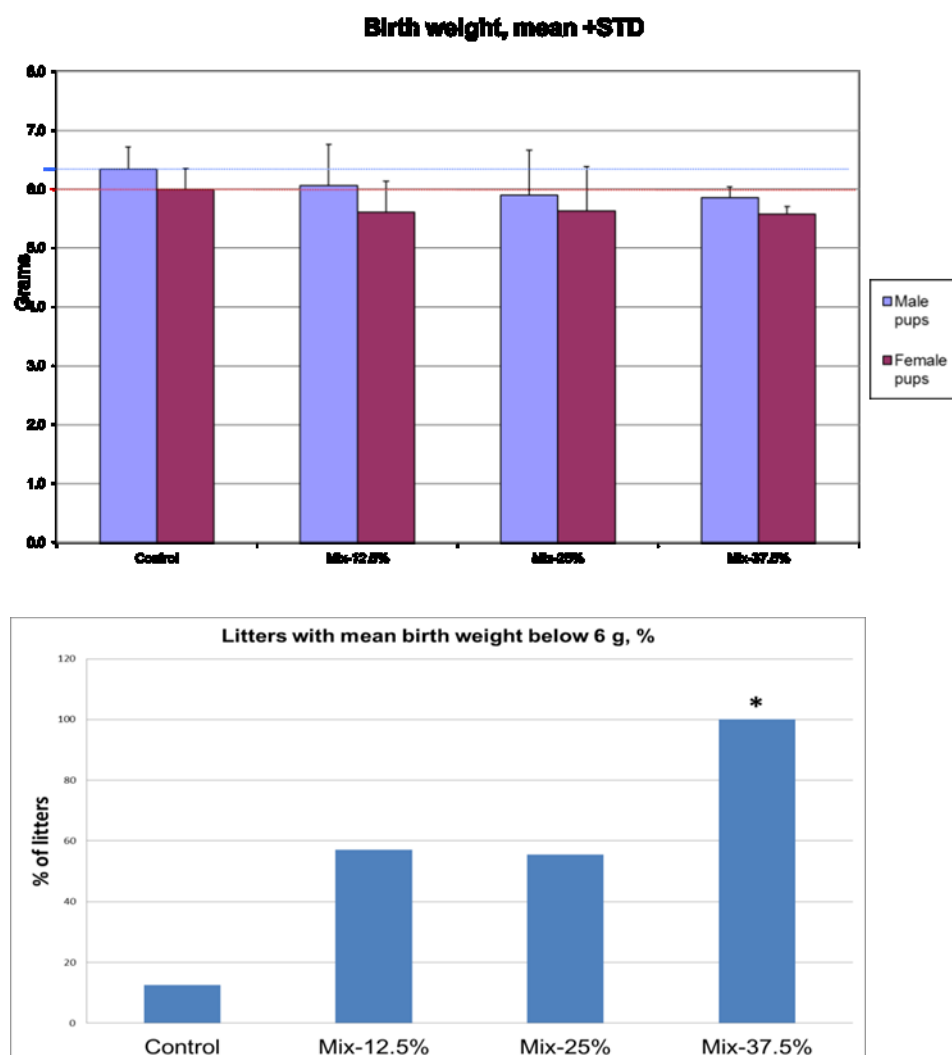


Figure 3. Birth weight in the range-finding study, mean+STD (top) and % litters with mean birth weight below 6 g (bottom), N= 8, 7, 9, 6 (number of litters)

AGD and NR

The AGD (analysed with body weight as covariate) was decreased at all doses in both male and female pups (Figure 4, Table 9) with $p < 0.0001$ for the males and $p \leq 0.001$ for the females. Nipple retention in the male pups appeared slightly higher than controls at Mix-37.5% (Table 9). However, the marginal increase only reflected few male pups with a few additional nipples and there were no statistically significant differences ($p > 0.26$, Fisher's Exact Test). The number of nipples in female pups were unaffected by the exposure.

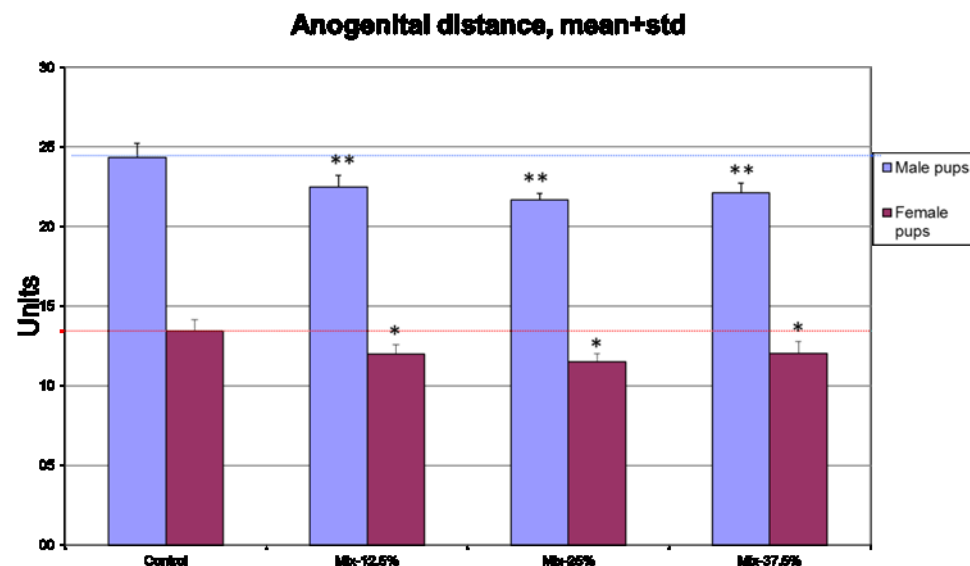


Figure 4. Effects of pesticide mixture on AGD in prenatally exposed male and female rats on the day after delivery in the range-finding study, results are shown as mean units +STD, N= 8, 7, 9, 6 (Number of litters) (1 unit= 0.161mm). Statistical significance is indicated by *, with $p < 0.001$, ** with $p < 0.0001$. AGD was decreased at all doses in both male and female pups.

Section on PD 16

The body weights of the dams were similar among groups, whereas the absolute and relative liver weight was significantly increased in the group exposed to the highest dose of Mix-37.5% (Figure 5).

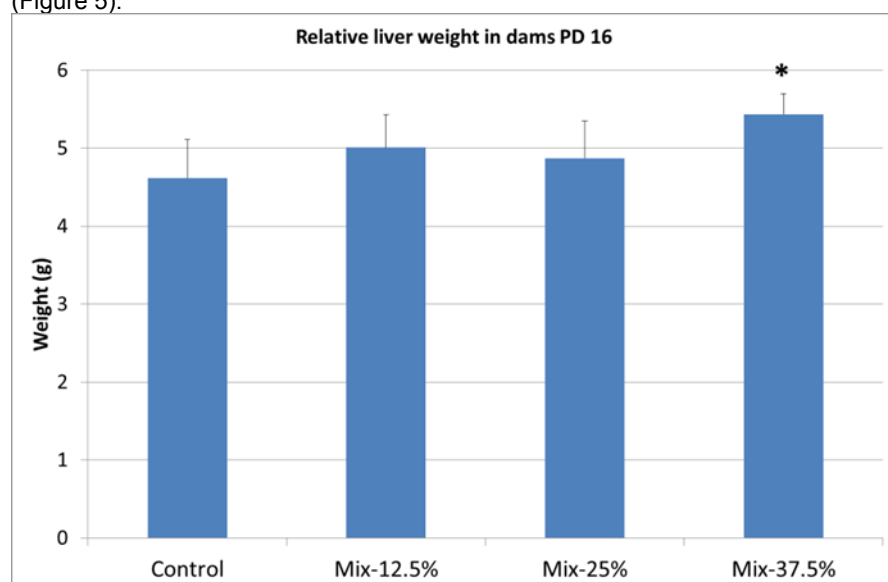


Figure 5. Relative liver weight in dams PD 16 in the range-finding study, g per 100 g body weight, mean+STD, N= 8, 7, 9, 6

Metabolic Biomarkers

Examination of effects on the levels of metabolic hormones related to metabolic syndrome and obesity were done simultaneously for the 7 following biomarkers: Amylin (active), C-peptide, GLP-1, Glucagon, Insulin, Leptin, and TNF α , whereas blood glucose levels were analysed separately.

A significant increase in glucagon in the male offspring was observed in the highest dose group (37.5%) (Figure 7). For the plasma levels of insulin, leptin, C-peptide and GLP-1 no significant effects were found either in offspring or dams (Figure 6).

For the measure of Amylin (active) and TNF α , the levels in the samples from both dams and offspring were under the limit of detection, and for GLP-1 the levels were only measurable in the dams, and here no significant effects were seen.

The level of glucose in blood on PD 16 was significantly decreased in the dams exposed to the highest dose, i.e. Mix 37.5% (Figure 7, top).

The glucose levels in the pups on PD 16 appeared dose-relatedly increased, but the differences were only statistically significant in the male offspring exposed to the highest dose, i.e. Mix-37.5% (Figure 7, bottom). The glucose levels also appeared increased in female offspring from the high dose group, but the difference from controls was not statistically significant.

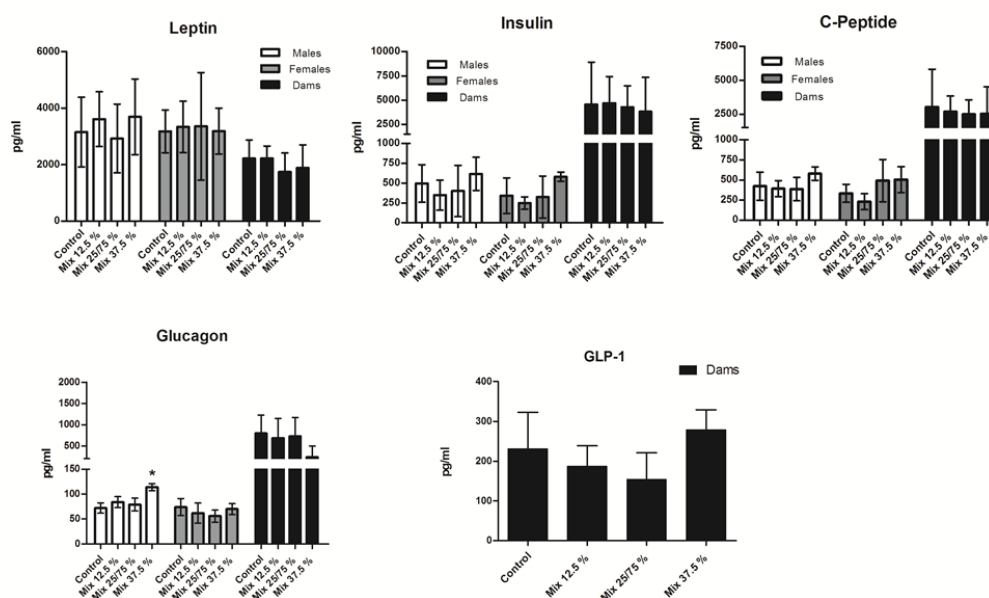


Figure 6. Metabolic biomarkers in dams and pups on PD 16 in the range-finding study.

Data represents mean \pm STD. N= 5-9. * Statistically significantly different from control (p<0.05)

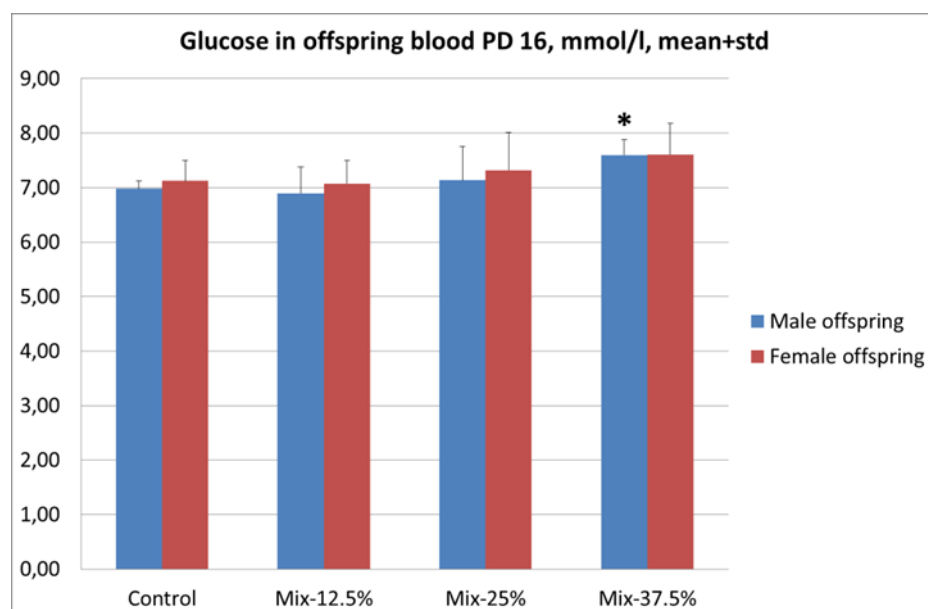
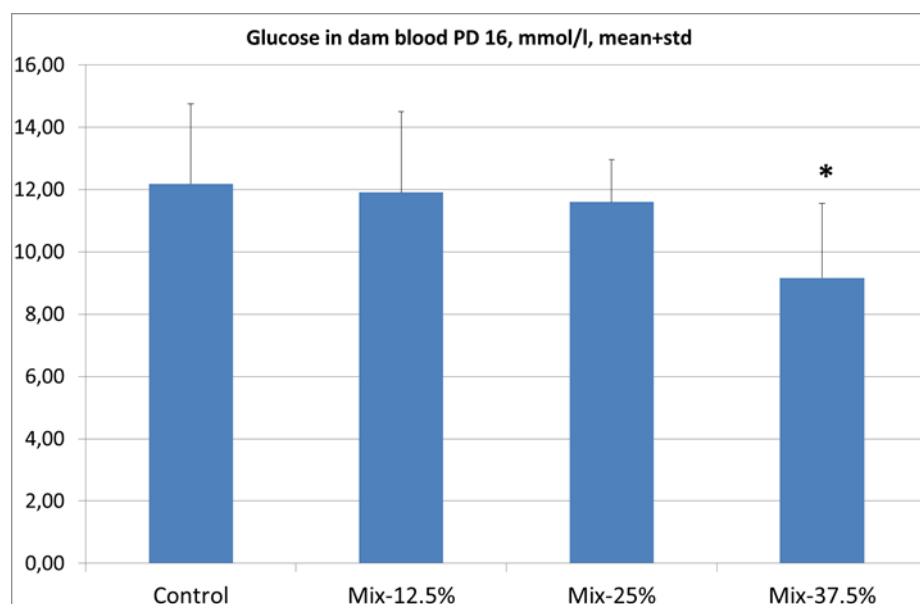


Figure 7. Glucose in blood samples from dams (top) and offspring (bottom), PD 16 in the range-finding study. Data represents mean+STD. N= 6-9. * Statistically significantly different from control (P<0.05)

3.2.4 Discussion of range-finding results

The main purpose of the range-finding study was to obtain data allowing selection of relevant dose levels for the large developmental toxicity mixture study in WP4. The following is focused on the four main questions that were addressed by this range-finding study:

1. Does the mixture dose levels chosen based on predictions of mixture effects on birth weight cause marked toxicity to the dams or marked effects on pregnancy parameters such as litter size and pup survival?
2. Does the mixture exposure seem to cause mixture effects on birth weight?
3. Does the mixture exposure seem to affect biomarkers for obesity?
4. Does the mixture exposure induce other relevant developmental toxicity effects, incl. effects on sexual development?

The group sizes were relatively small as the study was a range-finding study. Thus for some endpoints, the variation was quite high and the sensitivity for detecting effects was low. Therefore, lack of statistically significant effects was generally not be interpreted as absence of effects based on the results of this range-finding study.

Toxicity to dams and effects on pregnancy parameters

Already after two days of dosing, weight loss and symptoms of toxic effects were seen in the group exposed to the highest dose of Mix-75% and the dose was decreased to Mix-25% from GD 9. Thus, exposure to Mix-75% caused marked maternal toxicity and was too high a dose for the main study.

Some signs of maternal effects were also seen in the group exposed to Mix-37.5% in the form of slightly decreased mean maternal weight gain, increased relative liver weight and one dam showing symptoms of toxicity. For the one dam with symptoms this could be considered as marked toxicity, but for the other dams only signs of slight maternal toxicity were seen. Also, there were no effects on gestation length, litter size or pup survival. Based on these findings, it was evaluated that the highest dose level in the main study could be Mix-37.5% or lower, but should not be higher.

Mixture effect on birth weight

The individual pesticides were present in the mixture at lower doses than those calculated to lead to a detectable decrease of birth weight, i.e. less than 3% decrease. The exposure to the mixture seemed to cause a dose-related decrease of the mean birth weight. The differences were not significantly different from control values when analysing mean birth weight, however, significantly more litters with low mean birth weight (i.e. <6 gram) were found in the group exposed to Mix-37.5%. The observed effect of the mixture on mean birth weight as well as the predicted mixture effects based on dose-addition, independent action and no-mixture effect is shown in Figure 8. The results indicate mixture effect as the decrease in birth weights appears larger after the exposure to all off the mixtures doses compared to the predicted effect of the single pesticides. At the low and middle dose of the mixture the mixture effect predicted effect based on dose-addition and independent action appears similar to the observed effect. At the highest dose, both predictions models seem to overestimate the mixture effect. As the results are based on a limited number of litters per group, no clear conclusions with regards to mixture effects are drawn, but Mix-37.5% was evaluated as very relevant as the highest dose level in the main study.

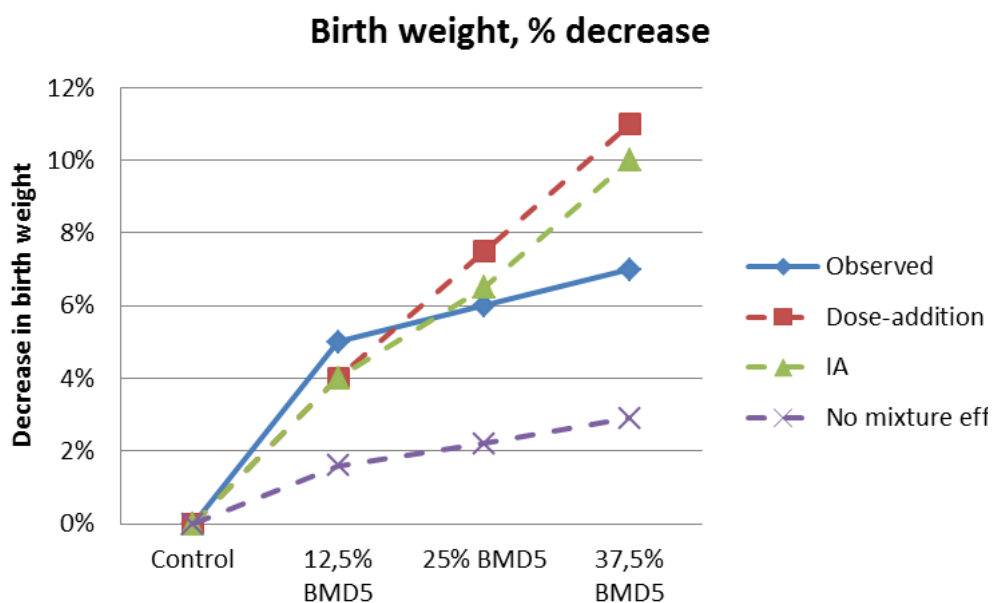


Figure 8. Birth weight decrease in % in the range-finding study. Observed results and predicted results based on dose-addition, independent action (IA) and no mixture effect, i.e. the predicted decrease induced by the single pesticides with the largest effect.

Biomarkers for obesity

The investigation of the effect of pesticide exposure on biomarkers related to metabolic syndrome, obesity and diabetes showed increased plasma glucagon and blood glucose levels in the male offspring 16 days of age, from the Mix-37.5% group. Previous studies on prenatal exposure to environmental chemicals and effects on obesity or obesity related endpoints, have shown that some effects first appear later in life i.e. in adulthood. It is possible that the lack of effects on most of the investigated biomarkers was because the effects are not evident at the measured age of the animals, but only in adulthood.

In contrast to the male offspring, the level of glucose in dam blood on PD 16 was significantly decreased in the Mix 37.5% group. This might indicate that the dams have transferred more glucose via the milk to pups at this dose level. Alternatively, it might reflect that the effect differ depending on the age where the animal is exposed, i.e. during development or during adulthood.

Other developmental toxicity effects, i.e. effects on sexual development

AGD was significantly decreased at all doses in both male and female pups. The effect on AGD was not due to the effect on birth weight as the statistical analysis corrected for body weight. Nipple retention in the male pups in the Mix-37.5% group appeared only marginally higher than controls and the difference was not statistically significant. The decreased AGD at birth indicates that the pesticide mixture affected the sexual development of the pups. Decreased AGD has previously been seen in both male and female offspring after exposure to endocrine disruptors with anti-androgenic or oestrogenic activity. After exposure to anti-androgenic compounds, nipple retention in the male offspring is usually also affected at the dose levels causing reductions in AGD, and nipple retention may actually be even more sensitive than AGD. On the other hand, it is our experience that decreased AGD in males and females combined with lack of clear effect on nipple retention in the male offspring could indicate oestrogenic activity of the pesticide mixture.

The 6 pesticides included in the pesticide mixture were evaluated in the EFSA report Identification of Cumulative Assessment Groups of Pesticides (<http://www.efsa.europa.eu/en/supporting/doc/269e.pdf>). Three of the pesticides (MCPB, quinalamine and thiram) are placed in a common assessment group for anti-androgen effect based on in vivo findings in the Draft Assessment Reports. The findings include decreased testes and prostate weight, histological changes of testes and inhibition of spermatogenesis. One of the other pesticides in the mixture (pirimicarb) is shown to be an aromatase inducer in vitro. Therefore, it seemed plausible that the effect of the pesticide mixture on AGD could be due to endocrine disrupting effects via anti-androgenic or oestrogenic mode of action. However, further data were needed before any clear conclusions could be reached.

The significant effect on both male and female AGDs, at all doses incl. the lowest dose, were considered as very relevant when considering the endpoints to be included in the main study. Thus, the data indicated that the target organs to be included in the main study should include reproductive organs shown to be most sensitive to endocrine disruption. Based on our long experience with studies of anti-androgens and oestrogens, we evaluated that the following organs would be the most relevant: testes, prostate and mammary gland. It would also have been relevant to consider assessment of effect on sperm count, as this is a severe effect that can be induced by both anti-androgens and oestrogens. However, further funding would have been needed to include this endpoint and this was not obtained.

3.2.5 Conclusions and proposals for the design of main study

The results of the range-finding study lead to the following conclusions:

- The highest dose of Mix-75% used initially lead to marked toxicity to the dams.
- This dose was therefore too high to use in the main study.
- The dose level Mix-37.5% lead to some signs of maternal effects, but no effects on gestation length, litter size or pup survival. The highest dose level in the main study could be Mix-37.5%.
- The exposure caused mixture effect on birth weight and significantly more litters with low mean birth weight were present in the group exposed to Mix-37.5%. Thus, based on the range-finding study Mix-37.5% was evaluated as a very relevant dose to include as the highest dose level in the main study.
- The investigation of the effect on biomarkers related to metabolic syndrome, obesity and diabetes showed increased plasma glucagon and glucose level in the male offspring at 16 days of age in the group exposed to Mix-37.5%. In the same group, increased glucose level was found in the dams. Thus, the planned further studies of biomarkers in the main studies appeared very relevant. Also, the target organs should include organs of relevance with regards to metabolic syndrome, i.e. pancreas, fatty tissue and liver.
- The AGD was significantly decreased at all doses in both male and female pups, indicating endocrine disrupting effect on sexual development. The target organs to be included in the main study should therefore include the reproductive organs shown to be most sensitive to endocrine disruption, e.g. prostate, testes and mammary gland.

3.3 Developmental toxicity mixture study

3.3.1 Introduction

The main purpose was to obtain data and results in a large scale developmental toxicity mixture study based on the findings in the range-finding study.

The specific questions addressed in the study were:

- Does the pesticide exposure cause mixture effects on birth weight?
- Does the mixture exposure affect biomarkers for obesity?
- Does the mixture exposure induce other relevant developmental toxicity effects, incl. effects on sexual development?

Thus, a large developmental mixture study in time-mated Wistar rats using Mix-37.5% as the highest dose of three doses was designed.

The number of animals per group in the developmental toxicity mixture study was comparable to the number of animals normally used in regulatory guideline studies for reproductive toxicity, i.e. 22 time-mated female rats expected to lead to around 20 litters per group. The guideline studies have been designed to have sufficient power for detecting effect sizes of regulatory relevance. For the endpoint birth weight our extensive experience shows that an effect size of 8% can be detected reliably and this is supported by power analysis. For the biomarkers for obesity, power analysis based on limited results from our earlier studies indicates that we may detect an effect size of 15-30%. Table 10 shows an overview of the study and table 11 shows details concerning the assessment of effects, i.e. specific endpoints and age of the animals.

Table 10 Overview of the developmental toxicity mixture study, study design and effects

| Study design | Effects in adults/dams | Effects in off-spring, pup day 1-24 | Effects in off-spring after pup day 24 to around 3 months |
|--|---|---|--|
| Developmental toxicity mixture study, pregnant animals, control, 3 doses, N = 22 time-mated rats | Body weight gain during pregnancy and lactation, gestation length, litter size, liver weight, | Birth weight, growth and survival, AGD and nipple retention, biomarkers for obesity, weight and histopathology of target organs | Body weight gain, food consumption, sexual maturation, biomarkers for obesity, weight and histopathology of target organs, gene expression |

Table 11 Effects assessed in the developmental toxicity mixture study, specific endpoints and time/age for the assessment of the endpoints

| Effect | Endpoints and time/age |
|---|--|
| Maternal toxicity | Signs of toxicity and body weight during pregnancy and lactation; GD 7-21 and PD 1-24 |
| Developmental toxicity, before weaning | Birth weight; PD 1 Pup growth and survival; PD 1, 6, 14, 24 AGD; PD 1 Nipple retention; PD 14 Blood samples for biomarkers for obesity; PD 16 Weight and histopathology of target organs (pancreas, the retroperitoneal fat pad, the femoral muscle, spleen and liver); PD 16 Weight and histopathology of target organs(pancreas, mammary tissue); PD 24 |
| Developmental toxicity, after weaning PD 24 | Body weight gain; ~1 per month and at sexual maturation Sexual maturation, females; from PD 27 to all positive (~PD 40) Sexual maturation, males; from PD 34 to positive (~PD 48) Food consumption; ~PM 5 Oral glucose tolerance test; ~PM 5 Blood samples for biomarkers for obesity; ~PM 5-6 Weight and histopathology of target organs (Liver and retroperitoneal fat in males and females as well as prostate and epididymal fat in males) ; ~PM 5-6 |

GD = Gestation day; PD = pup day; PM = Pup month

3.3.2 Materials and methods

Animals and dosing

The animal experiment was carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

Four groups of 22 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of the mixture of the 6 pesticides from gestation day (GD) 7 to pup day (PD) 16. Thus, the offspring were indirectly dosed via the placenta during the prenatal development and via maternal milk after birth. The mixture ratio was based on the estimated BMDs for 5% decreased birth weight for the individual pesticides. The mixture doses were of 0, 28, 90 and 210 mg/kg/day, i.e. 0%, 5%, 16% and 37.5% of this mixture (Table 12). The doses of the individual pesticides and the sum of them, i.e. the mixture doses, are shown in table 1. The names used for the four groups throughout this report will be: control, Mix-5%, Mix-16% and Mix-37.5%.

The substances used were corn oil (vehicle) (Sigma-Aldrich, Brøndby, Denmark), and cyromazine, MCPB, pirimicarb, quinoxaline, thiram and ziram. All pesticides were purchased in a technical quality from VWR- Bie & Berntsen, Herlev, Denmark.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Techniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lyngby, Denmark) and Tapvei Arcade 17 (Aspen wood)

shelters (Brogaarden). The cages were situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lux, temperature $21 \pm 2^{\circ}\text{C}$, humidity $50\% \pm 5\%$, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided ad libitum.

Table 12 Composition of pesticide mixture used for the developmental toxicity mixture in pregnant female animals (doses in mg/kg bw/day)

| Pesticide | Control | Mix-5% | Mix-16% | Mix-37.5% |
|-------------------|---------|--------|---------|-----------|
| Cyromazine | 0 | 17.5 | 64.8 | 131.25 |
| MCPB | 0 | 5.0 | 18.5 | 37.50 |
| Pirimicarb | 0 | 3.0 | 11.1 | 22.50 |
| Quinoclamine | 0 | 0.5 | 1.9 | 3.75 |
| Thiram | 0 | 1.0 | 3.7 | 7.50 |
| Ziram | 0 | 1.0 | 3.7 | 7.50 |
| Pesticide mixture | 0 | 28 | 90 | 210 |

Pregnancy and lactation

The animals were observed twice daily for signs of toxicity and dam body weights were recorded daily during pregnancy and lactation, i.e. from GD 7-21 and PD 1-16 and on PD 24.

The expected day of delivery, GD 23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as most of the animals gave birth on GD 23.

The weights of dams and individual pups were recorded after delivery and the pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The endpoints also included gestation length, number of implantation scars in the uterus of the dam on PD 24, body weights of pups at PDs 6, 14 and 24, anogenital distances in the new-born pups and nipple retention PD 14.

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in the offspring at the registration of birth using an ocular stereomicroscope. On PD 14, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring.

Section on PD 16

One male and one female pup were randomly selected from each litter, when possible. The offspring were weighed and decapitated. Blood samples were collected in 4 ml vacutainers with sodium heparin for analysis of biomarkers for obesity and analysis of glucose level. The internal organs of offspring were investigated macroscopically.

The retroperitoneal fat pad, the femoral muscle (musculus biceps femoris), spleen and liver were excised, cleaned and weighed. All organs (in case of the liver a piece of one of the lobes) were frozen and stored at -80°C for gene- or protein analysis. Furthermore, a piece of one of the liver lobes was placed in RNAlater and two standard sections from the liver were placed in formalin for histology. The pancreas was also excised from males and females and placed either in formalin for histology or in RNAlater for gene expression analysis. Tissues to be stored frozen were placed in liquid nitrogen and stored at -80°C . Tissues for histology were fixed in 4% formalin overnight.

Section on PD 24

Dams were decapitated in CO₂/O₂ anaesthesia and the numbers of uterine implantations were counted. The liver was examined macroscopically, excised and the weight was recorded.

The remaining pups were decapitated in CO₂/O₂ anaesthesia. Blood samples were collected in 10 ml vacuettes with sodium heparin for analysis of biomarkers for obesity, if possible and relevant. From one male and one female randomly selected from each litter per litter mammary tissues were taken for whole mount analysis, and pancreas were excised for histological studies if possible and relevant.

Section, adult offspring, 5-6 months old

The retroperitoneal fat pad and liver were excised from male and female pups, cleaned and weighed. The retroperitoneal fat pad was placed in RNAlater. Several sections were taken from the liver and placed in formalin or RNAlater. Furthermore, pancreas was excised from males and females and were allocated to either histology or gene/protein analysis. In males, the epididymal fat pad and the prostate, in association with the vesicula seminalis but also the ventral prostate alone, were additionally dissected and weighed. The ventral and the dorsal prostates were fixed in formalin. Tissues for histology were fixed in 4% formalin overnight.

Blood samples

All blood samples were stored on ice until centrifugation within one hour. The samples were centrifuged at 4000 rpm, 4 degrees for 10 minutes and the supernatant were transferred to 2 CM tubes with 160µl in one of them and the rest in the other and frozen at -80 degrees.

Weaning of pups

Approximately one male and one female pup from each litter were weaned on PD 24. The weaned offspring was housed in pairs of the same sex and exposure status.

Onset of puberty

Onset of puberty was assessed by determining day of vaginal opening or the day of balano-preputial separation in weaned female and male offspring, respectively. Registrations were performed daily in females from PD 27 until vaginal opening was detected in all animals. Males were examined daily from PD 38 until the last male was positive. Age and body weight of the rats were recorded on the day where vaginal opening and balano-preputial separation was first observed.

Metabolic Biomarkers PD 16 and adult offspring

Plasma levels of a selection of metabolic hormones were measured using LUMINEX technology and the MILLIPLEX® MAP Rat Metabolic Bead panel kit (#RMHMAG-84K) from Millipore, (Billerica, MA, USA). This kit was used for the simultaneous quantification of multiple analytes including: C-peptide 2, GIP (Gastric Inhibitory Polypeptide), PYY (Pancreatic peptide YY) IL-6, Insulin, Leptin, and MCP-1. The measurements were performed on plasma samples from 4-5 months old male and female offspring, as well as male offspring PD16. All analyses were performed according to the manufacturer's protocol, applying a Luminex IS 100® (Luminex Corporation, Austin, TX, USA). Acquired fluorescence data were analysed using the Bioplex ver. 5.0 software (Bio-rad Laboratories; USA).

Plasma levels of insulin and glucagon were also measured using two separate ELISA kits from Mercodia (Rat Insulin article no. 10-1250-01 and Glucagon article no. 10-1281-01). The glucagon measurements were performed on plasma samples from UD16 as well as 5-6 months old male offspring, and fasting insulin was measured on 5 months old male and female offspring. All analyses were performed according to the manufacturer's protocol, applying an Enspire® Multilable Plate Reader (PerkinElmer) for measuring the absorbance readout.

Rat obesity-related gene array

The Rat Obesity RT2 ProfilerTM PCR Array (Qiagen; PARN-017ZA) was used to profile the expression of 84 genes simultaneously. Three retroperitoneal fat pad samples (2x male, 1x female) were randomly selected from each group: Control, Mix-5%, Mix-16% and Mix-37.5%. Total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen) including on-column DNase I treatment as per manufacturer's instructions. cDNA was synthesised from 1 µg total RNA using the recommended RT2 First Strand Kit as described by the manufacturer (Qiagen). Samples were prepared for 384-well format and run on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative transcript levels were determined by the comparative Ct-method using the Qiagen on-line Data Analysis Center.

RT-qPCR TaqMan analysis

The protocols were essentially as described previously (Svingen et al, 2015), with 500 ng total RNA used to synthesise cDNA. TaqMan Gene Expression Assays (Life Technologies) were: *Agpr* (Rn01431703), *Il1b* (Rn00580432), *Lep* (Rn00565158), *Lepr* (Rn01433205), *Nmb* (Rn01478123), *Nmbr* (Rn00680847), *Npy* (Rn01410145), *Npy1r* (Rn02769337), *Pparg* (Rn00440945), *Ppargc1a* (Rn00580241) and *Tnf* (Rn99999017). RT-qPCR experiments were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well format using 3 µl diluted (1:20) cDNA per reaction. Relative transcript abundance was calculated by the comparative Ct-method with the geometric mean of the reference genes *Hprt* (Rn01527840) and *Rpl13a* (Rn00821946).

Histology

Tissues for histology were stored in formalin overnight, sectioned (3 µm) and stained for histopathologic evaluation. One section of liver from one female and one male per litter from control and high-dose groups from PD 16 and adult offspring were stained with haematoxylin and eosin (H&E) and evaluated for hypertrophy, vacuolisation (macro- and microvesicular) and signs of presence of glycogen in hepatocytes.

For pancreatic tissue, one section of pancreas from one male or female per litter on PD 16 was stained with H&E. Four fields per slide were evaluated for 1) the area of pancreatic islets, 2) area of islets relative to the area of pancreatic tissue and 3) the number of small, medium and large sized islets per area of pancreatic tissue.

In PD 16 and adult offspring from control and high-dose groups, an estimation of the relative amount of β -cells (insulin positive) and α -cells (glucagon positive) was performed by point-counting in immunohistochemically stained pancreas. In one section per animal β -cells were stained with a rabbit anti-insulin antibody (1:400 dilution, sc-9168, Santa Cruz Biotechnology), and in another section per animal α -cells were stained with a rabbit anti-glucagon antibody (1:500 dilution, sc-13091, Santa Cruz Biotechnology). A weak counterstain with H&E was performed to be able to differentiate structures in the exocrine pancreas. Point counts of β -cells, α -cells and other pancreas tissue were performed in a grid of 384 points in a minimum of 7 fields per animal on PD 16 (20x magnification) and in a minimum of 10 fields per animal in adults (10x magnification). An average of 64 and 37 insulin-positive and 31 and 13 glucagon-positive points per animal were counted on PD 16 and in adults, respectively. The ratios between 1) β -cell area and area of remaining pancreas tissue, 2) α -cell area and area of remaining pancreas tissue, and 3) β -cell area and α -cell area were calculated. The measurement of areas and point counting in the pancreas sections was performed in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA).

Whole mounts

Mammary glands of male and female prepubertal (PD 24) offspring were dissected and spread on a glass slide. The mammary glands were fixed in 4% formalin buffer, stained with alum carmine, dehydrated in alcohol, cleared with xylene and mounted with a coverslip. Whole mounts were scanned in a flatbed scanner and measurements were performed in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). Mammary glands were evaluated for out-growth (outer area, elongation, transverse growth, longitudinal growth, distance to the lymph node and distance to the 5th gland) and the number of TEBs in zone C was counted.

Offspring body weight gain after weaning

Offspring were weighed at weaning, upon sexual maturation and approximately every 14 days beginning on PND 44 (block 2 from PND 34) and ending on 5-6 months of age. The results were analysed by repeated measures ANOVA. Specific growth rate (SGR) was determined as weight gain between two points in time divided by previous body weight [Ng et al. 2010] and was determined for 5 months of age where also feed consumption was measured.

Food consumption

Weaned offspring and their chow were weighed on 5 months of age, and the chow consumption and weight of the animals was again registered after 7 days. As the animals were housed pairwise, food consumption was registered as the joined consumption of the 2 animals (from same group) housed together ($n = 8-9$). However, cages with animals housed with littermates were not excluded in the analysis (to avoid litter effects). This would impair the power which additionally was reduced by some technical problems. Energy efficiency was determined as weight gain between two points in time divided by energy intake between the two points [Ng et al. 2010]. Food consumption was expressed as food intake per day per 100 g rat.

Glucose Tolerance test

Weaned offspring were subjected to an oral glucose tolerance test at 5 months of age. The animals were fasted overnight for 18 hrs (predominantly light-period) prior to testing. The animals were placed individually in clean cages with bedding material 30 min before test-start to allow for acclimatization to the new surroundings. Tongue blood was drawn at time -15 min. for fasting insulin determinations. At time 0 min. the animals were dosed by oral gavage with 2 g D-glucose/kg bw in the form of an aqueous solution of 500 mg/ml D-glucose. Blood glucose from the tail tip was measured at times -30, 0, 15, 30, 60, 90, and 180 min from time of glucose dosing. Measurements were performed with Accu-Chek Aviva Nano Blood Glucose Monitor system (60308052812) and teststrips (06453970016) from Roche, Roche Diagnostics GmbH, Mannheim, Germany. Area Under the Curve (AUC) baseline corrected to fasting blood glucose was calculated and compared. A HOMA-IR, homeostasis model assessment = $\text{fasting insulin (ng/ml)} \times \text{fasting glucose (mM)} / 22.5 \times 0.0417$ score was calculated.

Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were analysed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. Birth weights were analysed using the number of offspring per litter as covariate and AGD and organ weights were analysed using body weight as a covariate. Birth and pup weights were examined for departures from normality and homoscedasticity, but no indications for a rejection were found. All weight variables were analysed with litter size as covariate, and for pooling purposes normalized to the means of the female or male controls. AGD data were analysed with pup birth weights as covariate, and by the AGD-index, i.e. AGD divided by the cube root of body weight. Statistical analyses were always adjusted using litter as an independent, random and nested factor. The number of nipples (NR) was assumed to follow a binomial-distribution with a re-

sponse range between 0 and θ_{\max} , with θ_{\max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{\max} was decided by considering the global fit (information criterion of Schwarz). Litter effects on NR and over-dispersion in the data were accounted for by using Generalized Estimating Equations (GEE). Statistical significance were assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha = 5\%$, two-sided). These tests were chosen as they were already implemented in the SAS procedure PROC GENMOD which was used for all statistical analysis (SAS Institute Inc., Cary, NC). Liver histological scoring data were analysed with Fisher's exact test (2x2) whereas pancreas point counting data were analysed using a 2-sided t-test, as only control and high-dose groups were investigated histologically.

Whole mount data were analysed with an ANOVA with body weight as a covariate and a Dunnett's post-hoc test correcting for multiple comparisons. Data not fulfilling criteria for homogeneity of variance or normal distribution (i.e. the distance to the lymph node and the number of TEBs) were analysed using a non-parametric statistical test (Kruskal-Wallis) with a Dunn's post-hoc test correcting for multiple comparisons. Non-parametric analyses of whole mount data were performed in GraphPad Prism 5 (GraphPad Software, San Diego California USA).

Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$. All analyses were performed using SAS Enterprise Guide 4.3, SAS Institute Inc., Cary, NC, USA.

Statistical analysis of metabolic biomarker data was performed using Sigma Plot v.11.0 (Systat Software Inc.). Data were tested for normality and homogeneity of variance. Data were analysed by one-way analyses of variance (ANOVA) followed by Dunnett's post hoc test. Significance was judged at $p < 0.05$. Statistical analyses of organ weights were performed in SAS Enterprise Guide 3.0 and 4.3 (SAS Institute Inc., Cary, NC, USA). Absolute organ weights were analysed using an analysis of variance (ANOVA) with body weight as a covariate. Relative organ weights were additionally analysed with an ANOVA.

3.3.3 Results

Effects during gestation

No clinical signs of toxicity were observed in the animals before GD 21. Due to signs of early parturition on GD 21 (rat dams give birth on GD22-23), caesarean sections were performed on two animals (from block 2), both from Mix-5% group. As this occurred only in Mix-5%, these findings are not considered to be related to the exposure. Four litters were not continued due to small litter size (i.e. below 4 pups), one control, one mix-5% and 2 mix-16% (see table 13 with pregnancy and litter data).

The body weight gain from GD 7 to GD 21 in the animals giving birth was significantly decreased compared to control values in the groups dosed with Mix-16% and Mix-37.5% (Figure 9a). The decreased body weight gain at Mix-16% and Mix-37.5% was primarily due to decreased body weight gain during the first week of exposure from GD 7-14, as the body weight gain during the second week of exposure from GD 14-21 was only significantly decreased in the Mix-37.5% (figure 9b).

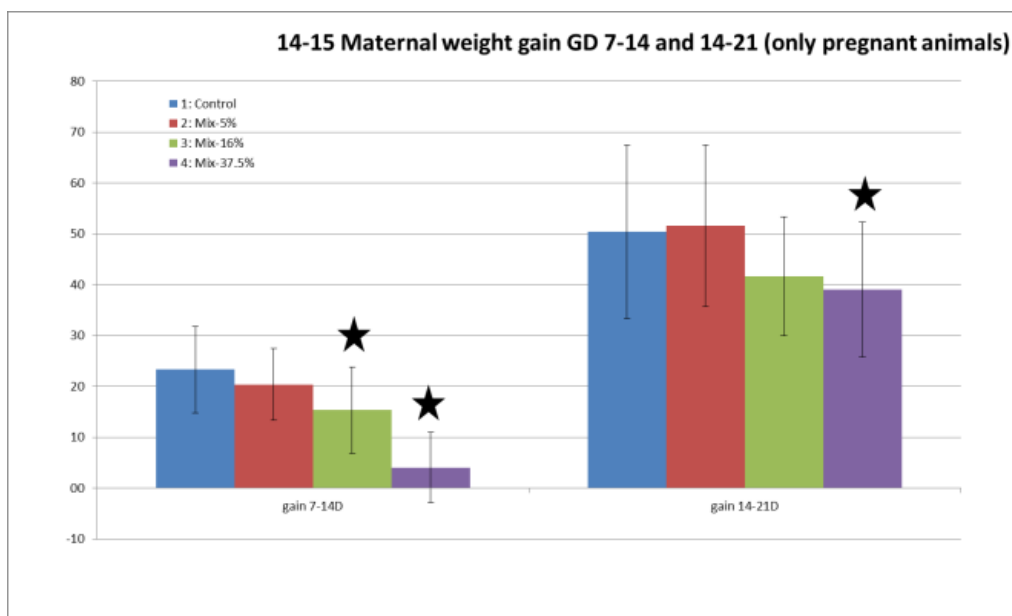
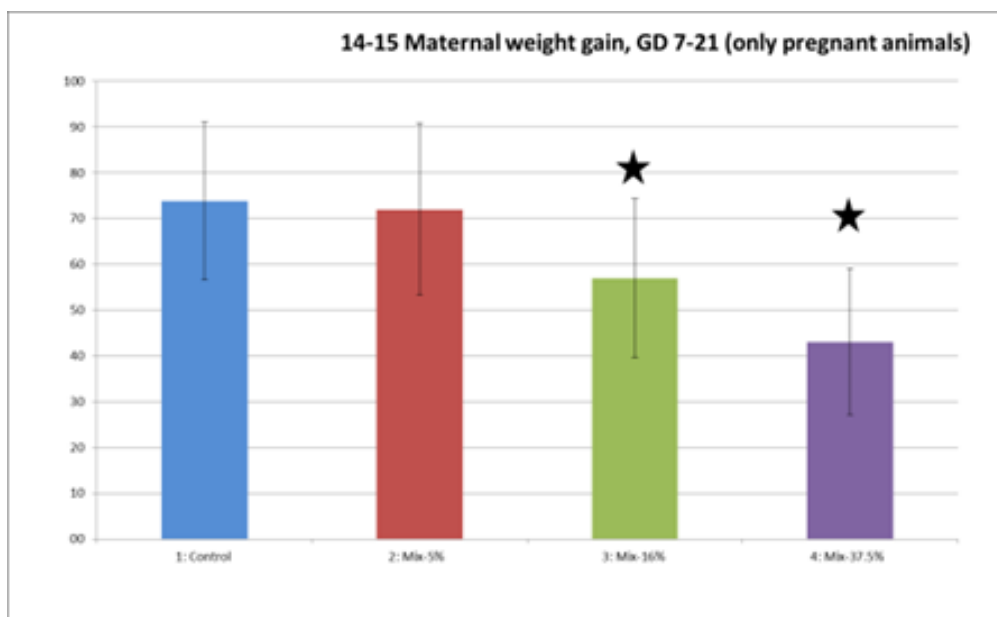


Figure 9a (top). Maternal body weight gain GD 7-21 in animals giving birth, mean \pm STD, N= 19-22, * $p < 0.05$

Figure 9b (bottom). Maternal body weight gain GD 7-14 and 14-21 in animals giving birth, mean \pm STD, N= 19-22, * $p < 0.05$

Pregnancy data and dam weights in the lactation period

Gestational length, number of live born pups per litter and the mortality of foetuses and pups were unaffected by the exposure.

The maternal body weight gain during the lactation period is shown in figure 10. From GD7 to PD 1 the weight gain was significantly decreased in the groups exposed to Mix-16% and Mix-37.5% ($p < 0.0001$). The maternal body weight gain from PD 1 to PD 24 appeared increased in the groups exposed to Mix-16% and Mix-37.5%, but the difference was only statistically significant in the Mix-37.5% group ($p = 0.009$).

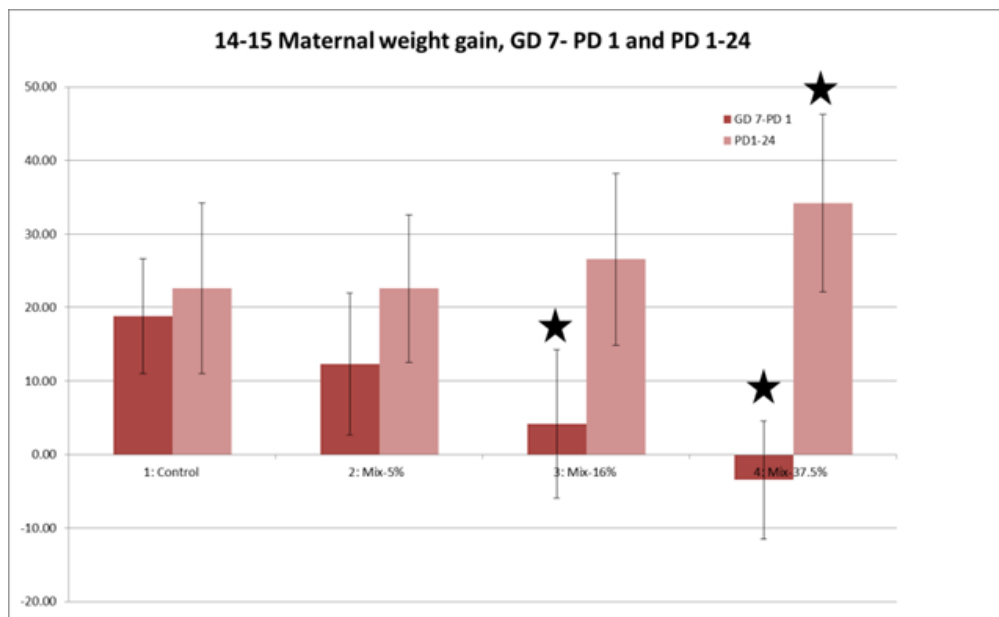


Figure 10. Maternal body weight gain GD 7-PD1 and PD1-24 in animals giving birth, mean±STD, N= 19-22 and 17-19 respectively. * p<0.05

The dam weight during the lactation period is shown in figure 11. Consistent with the body weight gain data, the groups exposed to Mix-16% and Mix-37.5% have lower body weights at the beginning, but in the last part of the lactation period (PD 14) only mix-37% showed significantly lower body weight and at PD 24 (one week after exposure was ended) none of the maternal weights were different.

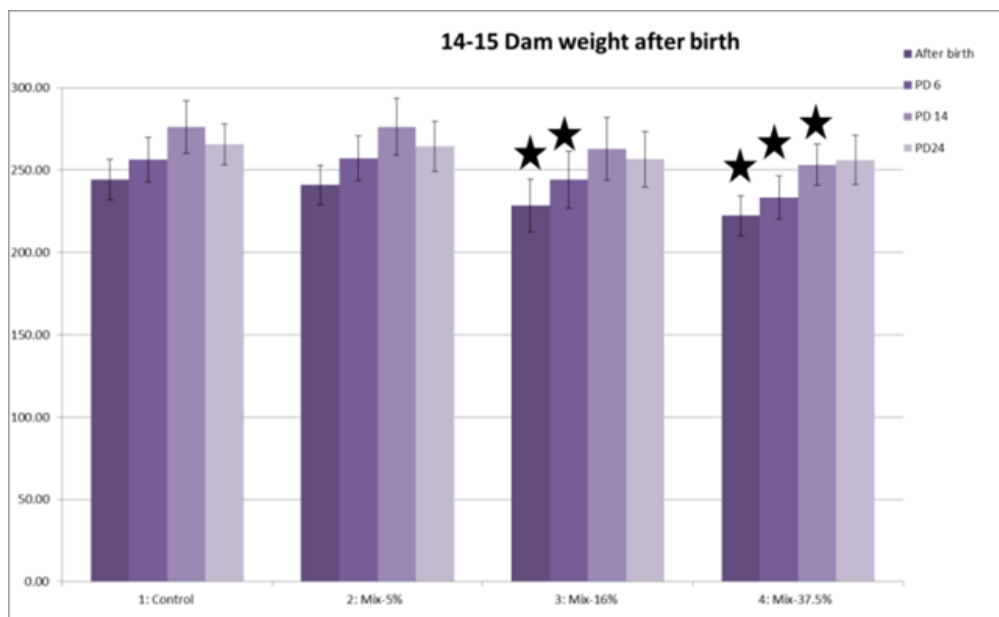


Figure 11. Maternal body weights during the lactation period, mean±STD, N= 17-19

Table 13 Pregnancy and litter dataData represent group means, based on dams or litter means \pm STD

| | Control | Mix-5% | Mix-16% | Mix-37.5% |
|--|-----------------|------------------|---|------------------|
| Time-mated females (no.) | 22 | 22 | 22 | 22 |
| Non-pregnant (no.) | 0 | 0 | 3 | 2 |
| Pregnant, but caesarean section | 0 | 2 | 0 | 0 |
| Pregnant, but no pups on PD1 (no.) | 2 | 0 | 0 | 1 |
| Pregnant, but not continued due to small litter size, i.e. below 4 (no.) | 1 | 1 | 2 | 0 |
| Pregnant, but early total litter loss after PD 1 (no.) | 1 | 0 | 0 | 0 |
| Females giving birth/litters (no.) | 21 | 20 | 19 | 20 |
| No. litters after PD 3 | 18 | 19 | 17 | 19 |
| Maternal bw GD7 (g) | 225.7 \pm 9.9 | 228.8 \pm 10.4 | 225.6 \pm 11.1 | 225.7 \pm 9.4 |
| Maternal bw gain GD7-GD21 (g) ^a | 73.8 \pm 17.2 | 72.0 \pm 18.7 | 57.0 \pm 17.4* | 43.1 \pm 16.0* |
| Maternal bw gain GD7- PD1 (g) ^a | 18.8 \pm 7.8 | 12.3 \pm 9.7 | 4.19 \pm 10.1* | -3.5 \pm 8.1* |
| Gestational length (d) | 23.1 \pm 0.3 | 23.0 \pm 0.5 | 23.1 \pm 0.2 | 23.1 \pm 0.4 |
| Litter size, live pups, PD 1 (no.) | 8.7 \pm 3.1 | 10.4 \pm 4.3 | 8.8 \pm 3.8 | 8.6 \pm 3.5 |
| Pup mortality, stillborn and dead after birth (%) | 1.3 \pm 5.7 | 0.0 \pm 0.0 | 5.3 \pm 22.9 (0.0 \pm 0.0 ^a) | 1.5 \pm 3.6 |
| Birth weight, male pups (g) | 6.7 \pm 0.45 | 6.2 \pm 0.55* | 6.1 \pm 0.62* | 5.7 \pm 0.41* |
| Birth weight, female pups (g) | 6.3 \pm 0.4 | 5.9 \pm 0.5 | 5.8 \pm 0.6* | 5.4 \pm 0.5* |
| AGD, males (units) | 21.7 \pm 1.4 | 21.9 \pm 1.5 | 21.6 \pm 1.0 | 22.4 \pm 1.2 |
| Nipple retention, male pups (no.) | 0.07 \pm 0.19 | 0.09 \pm 0.23 | 0.24 \pm 0.34 | 0.61 \pm 0.64* |
| AGD, female pups (units) | 10.7 \pm 0.6 | 11.0 \pm 0.7 | 11.1 \pm 0.8 | 11.1 \pm 0.8* |
| Body weight, PD 6, male pups (g) | 13.9 \pm 1.5 | 13.0 \pm 2.0 | 12.7 \pm 1.6* | 11.2 \pm 1.4* |
| Body weight, PD 6, female pups (g) | 13.3 \pm 1.4 | 12.6 \pm 1.7 | 12.3 \pm 1.5* | 11.0 \pm 1.3* |
| Body weight, PD 14, male pups (g) | 30.3 \pm 4.6 | 28.2 \pm 5.1 | 28.1 \pm 4.8 | 25.9 \pm 4.3* |
| Body weight, PD 14, female pups (g) | 29.8 \pm 4.3 | 28.0 \pm 5.3 | 27.5 \pm 4.5 | 25.9 \pm 4.8* |
| Body weight, PD 24, male pups (g) | 58.4 \pm 7.9 | 51.7 \pm 9.3 | 53.2 \pm 7.1 | 52.2 \pm 7.7* |
| Body weight, PD 24, female pups (g) | 56.8 \pm 7.6 | 51.7 \pm 8.9 | 51.8 \pm 7.6 | 51.6 \pm 7.6* |

* p < 0.05 ^aonly pregnant

- a) Excluding one litter with only 2 pups which died shortly after PD 1. It is common that litters with only 1-2 pups leads to insufficient maternal care, most likely due to insufficient stimulation of milk production

Birth weight and pup weight (Table 13, Figure 12)

The birth weights of the pups were dose-dependently decreased compared to control at all doses. In the Mix-37.5% group, the birth weight was 15% lower than control values, and the decreases were 6% and 9% in the groups exposed to Mix-5% and Mix-16%, respectively. The differences were significant in all of the dose groups apart from the mix-5% females. Pup body weights on PD 6 were similarly decreased as the birth weights, i.e. the decreases were 6%, 8% and 19% in the groups exposed to Mix-5%, Mix-16% and Mix-37.5%, respectively. However, the differences were only statistically significant at the two highest doses.

Pup body weights on PDs 14 and 24 appeared decreased at all doses by 6-10%, 7-8% and 10-14% in the groups exposed to Mix-5%, Mix-16% and Mix-37.5%, respectively. However, the differences were only statistically significant at the highest dose.

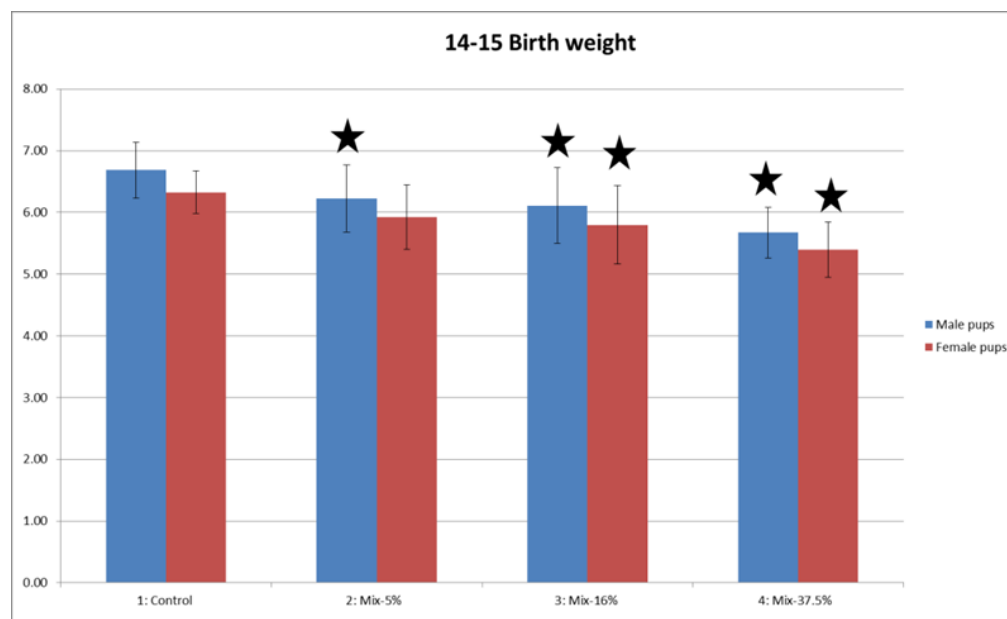


Figure 12. Birth weight, mean+STD, N= 19-20 (number of litters).

Mixture effect on birth weight

The observed effect of the mixture on mean birth weight as well as the predicted mixture effects based on dose-addition for both the range-finding and the main study is shown in Figure 13. The observed results at all three doses in the main study appear to be slightly more marked than predicted. However, these small differences are not of statistically significant. Thus, the predicted effect based on dose-addition corresponds well with the observed results for both studies. The predicted effect based on independent action (IA) is not shown as it was very similar to the DA-prediction (see fig. 8).

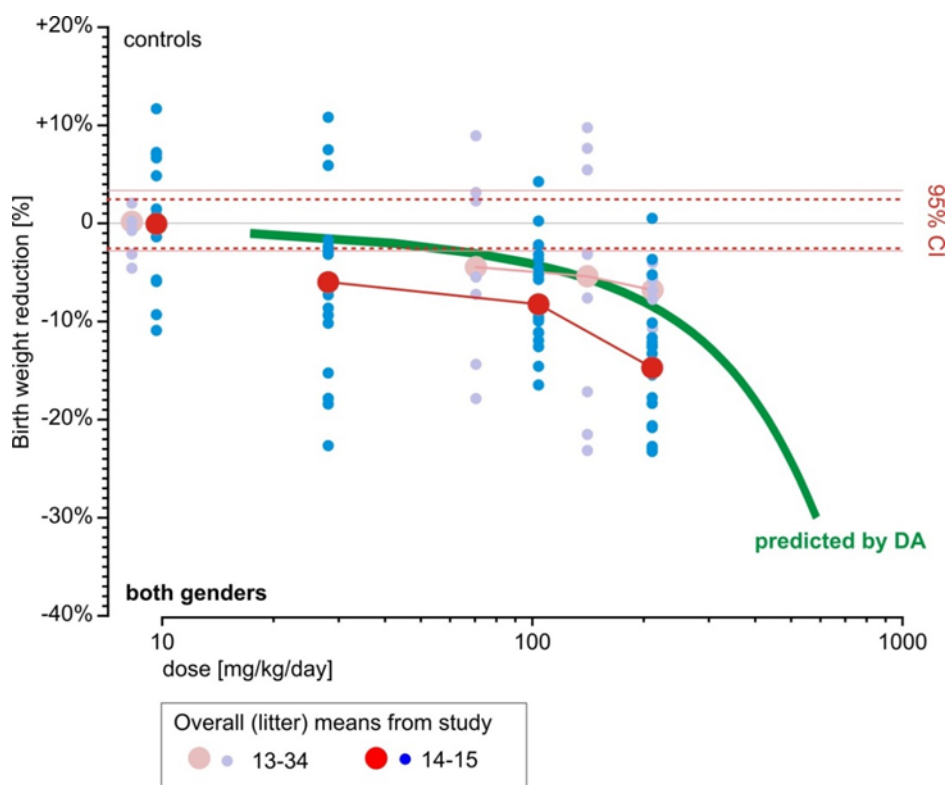


Figure 13. Birth weight decrease in % of controls in pups exposed to a mixture of six pesticides. Results shown are those observed in the range-finding study (study no.13-34) and the main study (study no.14-15) as well the predicted mixture results based on dose-addition (DA). The prediction curve is in line with the observed findings. The predicted effect based on independent action (IA) is not shown as it was very similar to the DA-prediction (see figure 8).

AGD and NR

The AGD in male offspring (analyse with body weight as covariate) was not affected by the exposure (Table 13). In female offspring, AGD was significantly longer at the highest dose level, i.e. Mix-37.5%.

Nipple retention in the male pups was significantly higher than controls at Mix-37.5% (Table 13). However, the increase was marginal and only reflected a few male pups with a few additional nipples.

The number of nipples in female pups were unaffected by the exposure (data not shown).

Organ weights and histology on PD 16

Organ weights of male and female offspring PD 16 are shown in table 14.

Body weights of offspring PD 16 were significantly decreased in both male and female offspring in the highest dose-group.

The relative weight of the retroperitoneal fat pad in males and females was increased although the body weights were decreased. It appears that the weight gain in adipose tissue relatively to the body weight gain was increased in the offspring in the highest dose group.

A large variation was present in liver weights in the Mix-5% dose-group, especially in the male offspring (Table 14 and Figure 14). Due to this difference, the variances were not normally distributed and were not possible to transform appropriately. However, when the Mix-5% was excluded from the statistical analysis the criteria of normal distribution were met and a statistically significant decrease in liver weights was seen in males in the Mix-37.5% group and in females in the Mix-16% and Mix-37.5% groups. The liver weights were then dose-dependently decreased but this decrease appeared to be related to smaller offspring rather than an exposure-related liver effect (Figure 14).

Table 14. Organ weights of PD 16 offspring. Absolute weights (abs) were analysed using an ANOVA with body weight as a covariate and Dunnett's test to determine group differences. Body weights and relative weights (rel) were analysed with an ANOVA and Dunnett's post hoc test. For the statistical analysis of liver weight the data were analysed both with and without the Mix-5% group. *p<0.05

| | Control | Mix-5% | Mix-16% | Mix-37.5% |
|-----------------------------|---------------|----------------|----------------|----------------|
| PD 16 males (abs) | N=18 | N=17 | N=17 | N=18 |
| Body weight (g) | 34 ± 5.3 | 30 ± 5.2 | 32 ± 5.4 | 29 ± 5.4* |
| Retroperitoneal fat pad (g) | 0.062 ± 0.020 | 0.055 ± 0.015 | 0.065 ± 0.018 | 0.068 ± 0.021 |
| Femoral muscle (g) | 0.060 ± 0.020 | 0.052 ± 0.015 | 0.052 ± 0.018 | 0.048 ± 0.018 |
| Spleen (g) | 0.126 ± 0.042 | 0.106 ± 0.035* | 0.123 ± 0.041 | 0.114 ± 0.037 |
| Liver (g) | 0.870 ± 0.148 | 0.819 ± 0.223 | 0.843 ± 0.149 | 0.793 ± 0.146* |
| PD 16 males (rel) | | | | |
| Retroperitoneal fat pad (g) | 0.181 ± 0.045 | 0.186 ± 0.047 | 0.205 ± 0.039 | 0.230 ± 0.053* |
| Femoral muscle (g) | 0.179 ± 0.064 | 0.176 ± 0.051 | 0.165 ± 0.048 | 0.165 ± 0.055 |
| Spleen (g) | 0.365 ± 0.072 | 0.349 ± 0.060 | 0.381 ± 0.064 | 0.385 ± 0.066 |
| Liver (g) | 2.557 ± 0.115 | 2.827 ± 0.893 | 2.657 ± 0.104* | 2.732 ± 0.154* |
| PD 16 females (abs) | N=18 | N=19 | N=14 | N=17 |
| Body weight (g) | 33 ± 5.0 | 32 ± 7.1 | 29 ± 4.0 | 29 ± 4.9* |
| Retroperitoneal fat pad (g) | 0.045 ± 0.017 | 0.049 ± 0.014 | 0.036 ± 0.008* | 0.046 ± 0.012 |
| Femoral muscle (g) | 0.053 ± 0.022 | 0.057 ± 0.026 | 0.046 ± 0.014 | 0.054 ± 0.018 |
| Spleen (g) | 0.128 ± 0.032 | 0.121 ± 0.055 | 0.118 ± 0.027 | 0.119 ± 0.036 |
| Liver (g) | 0.869 ± 0.140 | 0.786 ± 0.191* | 0.774 ± 0.112* | 0.803 ± 0.148* |
| PD 16 females (rel) | | | | |
| Retroperitoneal fat pad (g) | 0.132 ± 0.041 | 0.156 ± 0.025 | 0.125 ± 0.021 | 0.160 ± 0.034* |
| Femoral muscle (g) | 0.161 ± 0.072 | 0.181 ± 0.072 | 0.160 ± 0.059 | 0.191 ± 0.072 |
| Spleen (g) | 0.379 ± 0.052 | 0.369 ± 0.080 | 0.404 ± 0.049 | 0.407 ± 0.064 |
| Liver (g) | 2.592 ± 0.103 | 2.532 ± 0.427 | 2.657 ± 0.117 | 2.780 ± 0.115* |

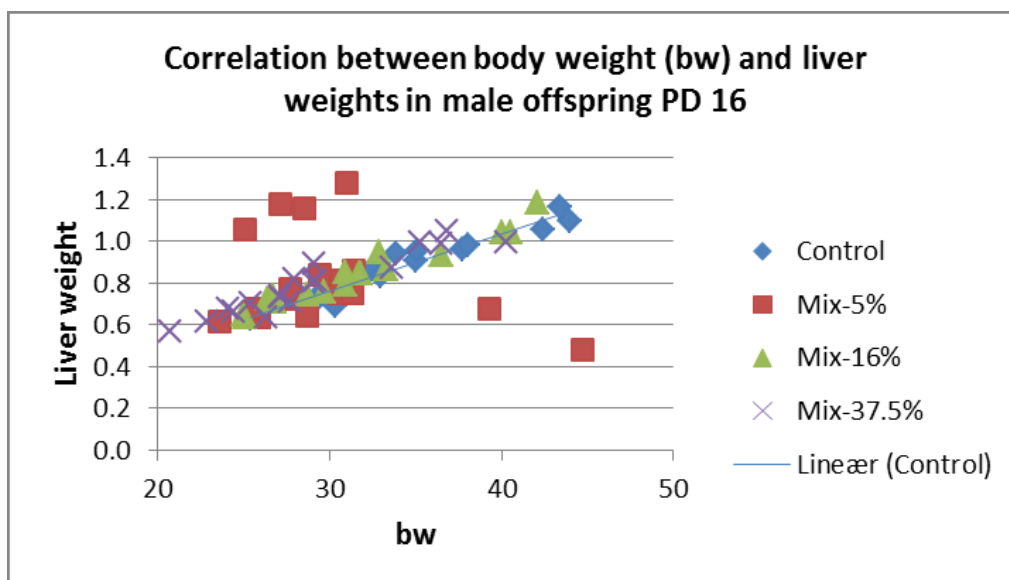


Figure 14. Correlation between liver weights and body weights in male offspring PD 16. A large variation in liver weights is present in the Mix-5% dose-group (red boxes). All other dose-groups showed good correlation between the liver weight and the body weight.

Decreased absolute organ weights (ANOVA with body weight as a covariate) were seen for the spleen in males in Mix-5% group and for the retroperitoneal fat pad in females in the Mix-16% group, but these changes were not dose-dependent and were not retrieved in relative organ weights. The changes observed in absolute weights of spleen and retroperitoneal fat pad appeared to be related to the lower body weights of offspring in the Mix-5% and Mix-16% groups, although body weights were not significantly lower than controls. No statistically significant changes in (either absolute or relative) organ weights were seen for the femoral muscle in males and females or for the spleen in females PD 16.

Liver histology of control and high-dose male and female offspring only showed slight changes PD 16. Male livers from the high-dose group appeared to have less glycogen in hepatocytes compared to controls. This was evident as the incidence of males with presence of small amounts of what was assumed as glycogen was decreased compared to controls (13 out of 19 control males and 2 out of 16 high-dose males; $p=0.002$ in a 2x2 Fisher's exact test). Such hepatic changes could reflect individual differences in food intake before euthanasia but the distribution between the two groups was surprising. However, a special staining, e.g. a Periodic acid-Schiff stain, should be used to verify if the changes observed indeed are related to glycogen. No hypertrophy or vacuolisation was observed in male livers. In female livers PD 16 changes resembling hydropic degeneration with slight enlargement of hepatocytes and bright cloudy cytoplasm without displacement of nucleus were observed ($p=0.03$, high dose compared to control). No differences in hypertrophy, glycogen accumulation or vacuolisation were observed in female livers.

Pancreas histology showed no changes in pancreatic islet area (absolute or relative to the total pancreatic area), the number of pancreatic islets relative to the area of pancreatic tissue or in the distribution of small, medium sized or large pancreatic islets. Histology of pancreas from offspring PD 16 showed no exposure related effects on the amount of glucagon or insulin producing cells in the pancreas or in the ratio between insulin- and glucagon producing cells (Figure 15).

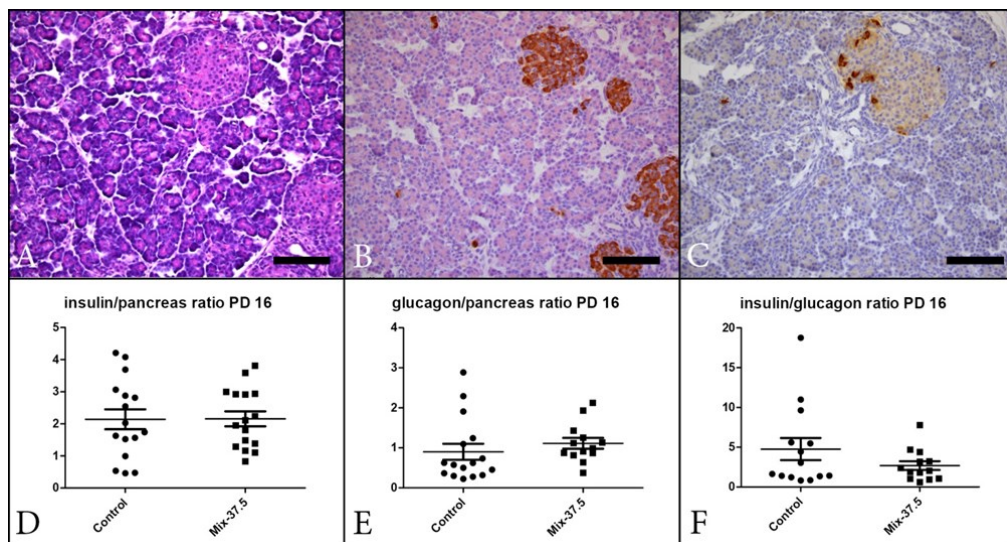


Figure 15. Morphometric analysis of pancreatic tissue in offspring PD 16. A-C: Pancreas from a control offspring on PD 16 stained with H&E (A) or stained immunohistochemically for insulin (B) or glucagon (C) with slight H&E background staining. No changes were found in the insulin/ pancreas ratio (D), the glucagon/ pancreas ratio (E) or in the ratio between insulin and glucagon producing cells (F) PD 16 . N= 13-14 per group . Black bars in pictures A-C represent 100 µm.

Section on PD 24

Female offspring PD 24 (one per litter) showed a decreased body weight in the lowest dose-group (Mix-5%). No other statistically significant changes were seen in body weights in dams or male offspring or in liver weights in dams (Table 15).

In mammary glands from male offspring, the distance to the lymph node was significantly increased in the lowest dose-group (Mix-5%) compared to controls (data not shown). No other statistically significant changes in measurements of outgrowth and development of mammary glands of male and female offspring PD 24 were found.

Table 15. Weights of dams and offspring at sacrifice on PD 24

| | Control | Mix-5% | Mix-16% | Mix-37.5% |
|------------------------|--------------|--------------|--------------|--------------|
| Dams (n) | 18 | 18 | 16 | 18 |
| Body weight (g) | 267 ± 10.8 | 265 ± 15.4 | 258 ± 16.2 | 256 ± 14.9 |
| Liver (g) | 12.47 ± 1.40 | 12.65 ± 1.95 | 12.36 ± 1.95 | 12.64 ± 2.11 |
| Relative liver | 4.66 ± 0.44 | 4.76 ± 0.63 | 4.78 ± 0.61 | 4.92 ± 0.69 |
| Male pups (n) | 15 | 16 | 15 | 15 |
| Body weight | 55 ± 7.4 | 50 ± 6.1 | 53 ± 7.0 | 51 ± 7.5 |
| Female pups (n) | 15 | 17 | 13 | 14 |
| Body weight | 55 ± 6.3 | 49 ± 6.9* | 51 ± 6.7 | 49 ± 6.0 |

Absolute weights were analysed using an ANOVA with body weight as a covariate. Body weights and relative weights were analysed with an ANOVA. * p<0.05

Table 16. Organ weights of adult offspring at sacrifice (4-5 months old)

| | Control | Mix-5% | Mix-16% | Mix-37.5% |
|-----------------------------|----------------|---------------|---------------|---------------|
| Males (abs) | N=18 | N=19 | N=14 | N=17 |
| Body weight (g) | 441 ± 42.2 | 421 ± 49.2 | 411 ± 47.9 | 410 ± 26.1 |
| Retroperitoneal fat pad (g) | 4.425 ± 1.799 | 4.100 ± 1.544 | 4.668 ± 1.607 | 4.677 ± 1.522 |
| Epididymal fat (g) | 3.864 ± 1.299 | 3.516 ± 1.125 | 4.074 ± 1.430 | 3.804 ± 1.209 |
| Prostate/Sem. vesicle (g) | 2.703 ± 0.333 | 2.855 ± 0.343 | 2.797 ± 0.408 | 2.605 ± 0.326 |
| Ventral prostate (g) | 0.667 ± 0.1222 | 0.693 ± 0.117 | 0.726 ± 0.166 | 0.647 ± 0.160 |
| Liver (g) | 12.08 ± 1.06 | 11.66 ± 2.15 | 11.48 ± 1.20 | 11.43 ± 1.08 |
| Males (rel) | N=18 | N=19 | N=14 | N=17 |
| Retroperitoneal fat pad (g) | 0.995 ± 0.347 | 0.961 ± 0.316 | 1.118 ± 0.320 | 1.139 ± 0.343 |
| Epididymal fat (g) | 0.870 ± 0.241 | 0.828 ± 0.221 | 0.988 ± 0.312 | 0.927 ± 0.282 |
| Prostate/Sem. vesicle (g) | 0.564 ± 0.205 | 0.647 ± 0.182 | 0.562 ± 0.276 | 0.604 ± 0.166 |
| Ventral prostate (g) | 0.153 ± 0.032 | 0.167 ± 0.033 | 0.179 ± 0.044 | 0.150 ± 0.052 |
| Liver (g) | 2.592 ± 0.103 | 2.532 ± 0.427 | 2.657 ± 0.117 | 2.780 ± 0.115 |
| Females (abs) | N=18 | N=19 | N=14 | N=17 |
| Body weight (g) | 242 ± 24.4 | 234 ± 18.5 | 232 ± 16.5 | 228 ± 16.8 |
| Retroperitoneal fat pad (g) | 1.486 ± 0.365 | 1.521 ± 0.392 | 1.575 ± 0.547 | 1.586 ± 0.353 |
| Liver (g) | 6.570 ± 0.949 | 6.375 ± 0.669 | 6.434 ± 0.706 | 6.209 ± 0.560 |
| Females (rel) | N=18 | N=19 | N=14 | N=17 |
| Retroperitoneal fat pad (g) | 0.611 ± 0.120 | 0.648 ± 0.150 | 0.672 ± 0.195 | 0.697 ± 0.153 |
| Liver (g) | 2.712 ± 0.239 | 2.727 ± 0.156 | 2.774 ± 0.271 | 2.728 ± 0.167 |

Absolute weights were analysed using an ANOVA with body weight as a covariate. Body weights and relative weights were analysed with an ANOVA. Sem. vesicle: seminal vesicle.

Section of 4-5 months old offspring

No significant changes in organ (absolute or relative) or body weights were found in offspring 4-5 months old (Table 16). The relative weight of the retroperitoneal fat pad in males was elevated in the highest dose-group (14.5% compared to controls), although not statistically significant. Histological evaluation of livers of male and female 4-5 months old offspring (controls and high-dose groups) showed no signs of hypertrophy, vacuolisation or glycogen accumulation changes.

Histology of pancreas from adult offspring showed slight decreases in the β -cells/pancreas ratio ($p=0.08$ in unpaired t-test) and the α -cells/pancreas ratio ($p=0.07$ in unpaired t-test) in the high-dose group compared to controls. However, one animal in the control group showed high ratios for both measurements. When this outlier was excluded from the dataset, no significant differences were seen for the ratios of insulin- or glucagon producing cells between the two groups. No difference was found in the ratio between β -cells and α -cells.

Onset of puberty

In males, the onset of puberty assessed by determining day of the day of balano-preputial separation was not affected by the exposure (Table 17). The body weight at onset of puberty was 6% lower in the groups exposed to Mix-5% and Mix-16% and by 8% in the Mix-37.5% group. These differences were significantly different from control in the high dose group ($p=0.005$) while Mix-5% and Mix-16% males only tended to be smaller ($p=6-7\%$)

In females, the onset of puberty assessed by determining day of vaginal opening appeared dose-relatedly delayed and was one day later in the group exposed to Mix-37.5%. This difference was not significantly different from control. The body weight at onset of puberty appeared to be decreased by 5% in the females in this group, but the difference was not statistically significant.

Table 17. Puberty data

| | Control | Mix-5% | Mix-16% | Mix-37.5% |
|-----------------------------------|------------------|------------------|------------------|-------------------|
| Males, onset of puberty (day) | 44.65 \pm 2.11 | 44.83 \pm 2.12 | 44.33 \pm 1.71 | 44.50 \pm 1.47 |
| Males, body weight at puberty (g) | 163.7 \pm 12.5 | 154.0 \pm 16.2 | 154.1 \pm 12.0 | 150.1 \pm 10.1* |
| Females, onset of puberty (day) | 32.60 \pm 1.96 | 33.17 \pm 1.62 | 33.56 \pm 2.68 | 33.61 \pm 1.65 |
| Females, body weight at puberty | 88.8 \pm 12.1 | 86.4 \pm 11.8 | 87.5 \pm 11.9 | 84.1 \pm 12.8 |

* $p < 0.05$

Metabolic Biomarkers

Biomarkers for obesity

The protein biomarkers for obesity were measured in male and female offspring at ages between 4-5 months and for male offspring also at UD16. A significant increase in leptin was observed in the female offspring age 4-5 month in the highest dose group (Mix 37.5%) (Figure16). For the other measured biomarkers no significant effects were found. A tendency towards dose dependent increase in C-peptide levels was seen in the female offspring, as well as a trend toward a decrease in Il-6 levels in the two highest dose groups also in the female offspring (Figure 17); however neither of these effects was statistically significant.

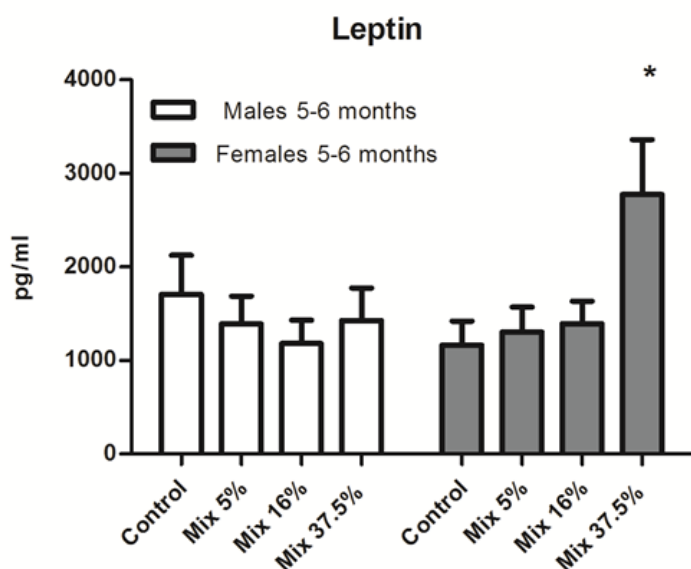


Figure 16: Leptin plasma levels in male and female offspring age 5-6 month. The results shown are from the main study. Data is presented as mean \pm SEM. * $p < 0.05$ by ANOVA with Dunnett's post hoc test. N= 17-20 for the males and N = 10-14 for the females. Especially for the females several samples were out of range which is why the sample number (N) is lower.

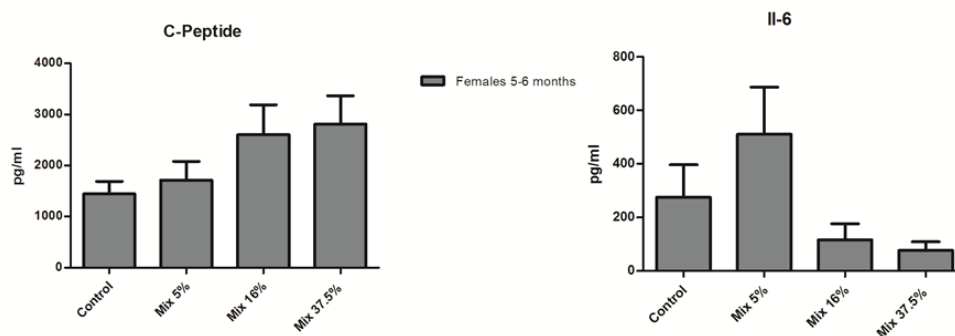


Figure 17: Plasma levels of C-peptide and IL-6 in female offspring age 5-6 month. The results shown are from the main study. Data is presented as mean \pm SEM. N= 12-19 for the C-Peptide measurements, and N = 18-20 for IL-6. There are no statistically significant findings.

In the male offspring UD 16, no effects were found on the measured biomarkers. In the prior range finding study a significant increase of 57% relative to controls was found on glucagon plasma levels in male offspring UD16 in the Mix 37.5% dose group. In the main study an increase of around 37 % relative to controls was also seen in the Mix 37.5% dose group, but it was not statistically significant (Fig. 18).

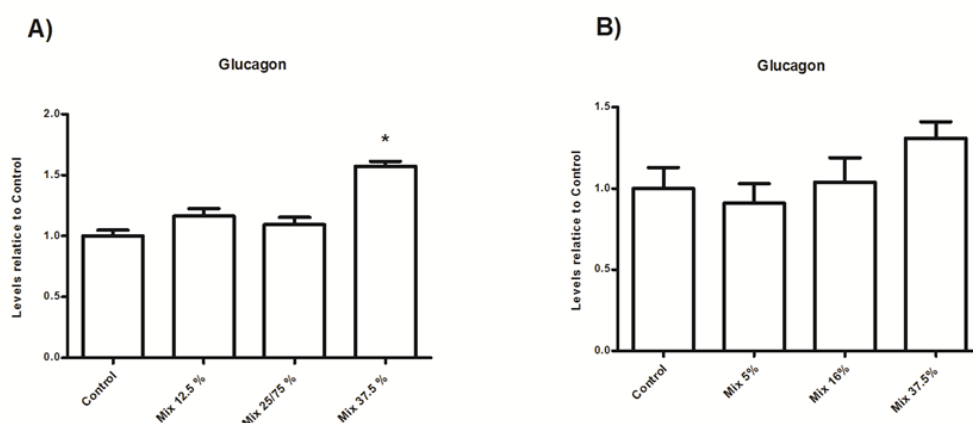


Figure 18: Glucagon plasma levels in male offspring UD16 found in A) the range finding study and B) main study. To improve comparison of data between the two studies data is presented in the form of normalized data relative to control and as mean \pm SEM. * $p < 0.05$ by ANOVA with Dunnett's post hoc test. N= 6-9 in the range finding study and N = 17-18 in the main study.

Fat tissue gene expression profiling 5-6 months of age

To gain insight into molecular effects on metabolism of rats following exposure to the pesticides, we performed a pilot 84-gene array on retroperitoneal fat tissues. Three samples, two male and one female, were selected from each of control and the three exposure groups (Mix-5%, Mix-16% and Mix-37.5%). As shown in Fig. 19, a few genes were dysregulated more than 2-fold in exposed animals relative to control. For instance, Nmb and Npy1r were upregulated in all exposure groups, whereas Lepr and Nmb were down-regulated.

Based on results from the pilot screen, and to verify its accuracy, additional RT-qPCR analyses were carried out on a larger sample size for eleven target genes. These genes all showed some degree of dysregulation in the expression screen and were thus tested across 16-17 individual tissue samples from each group; 8-9 males and 8 females. As evident in Fig. 20, the degree of dysregulation was less than what was observed initially, but the trends were similar. That is, genes displaying either up- or down-regulation in the RT-Profiler array correlated with up- or down-regulation by RT-qPCR analyses.

Since a noteworthy degree of sexually dimorphic transcript abundance was observed for some genes, the data were split into male and female groups. Although trends were observed, no statistically significant changes in gene expression was observed relative to control, either for male or female samples. Neither were any significant changes observed when all data were pooled together (data not shown). A significant difference in *Nmbr* transcript abundance was observed between male and female control tissues ($p=0.030$, with mRNA levels approximately 2.5-fold higher in female fat tissue). *Nmb* displayed a strong trend toward being more highly expressed in female fat tissue at 2.2-fold ($p=0.075$), whereas *Lepr* trended towards a higher expression in male fat tissue at 1.6-fold ($p=0.088$).

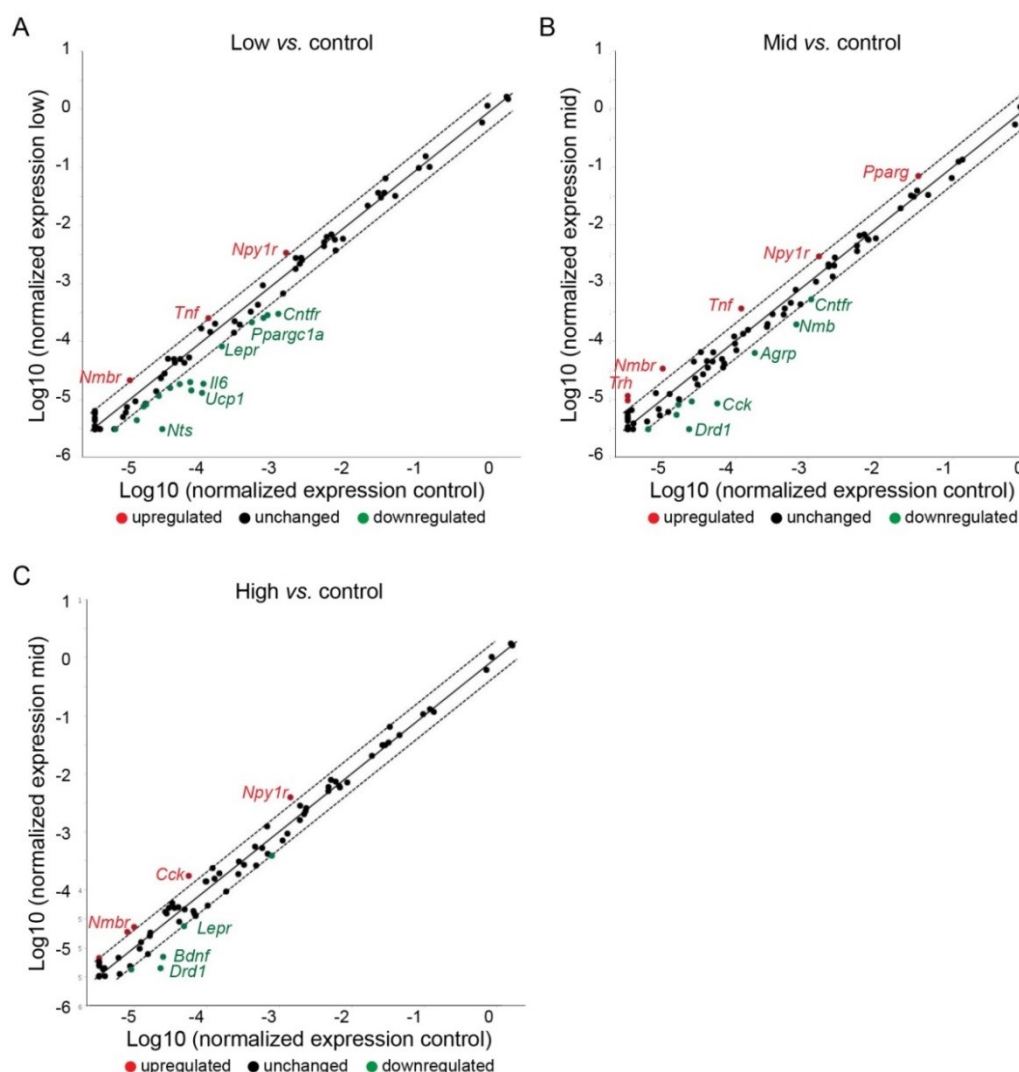


Figure 19. Obesity gene array on fat tissue from rats after exposure to a mixture of pesticides. Scatter-plots of differentially expressed obesity-related genes in retroperitoneal fat pads from control versus exposure groups: Mix-5% (A), Mix-16% (B) and Mix-37.5% (C). Relative fold expression was determined from average transcript abundance across groups ($n=3$ from each group). Genes up-regulated more than 2-fold are highlighted in red and genes down-regulated more than 2-fold highlighted in green.

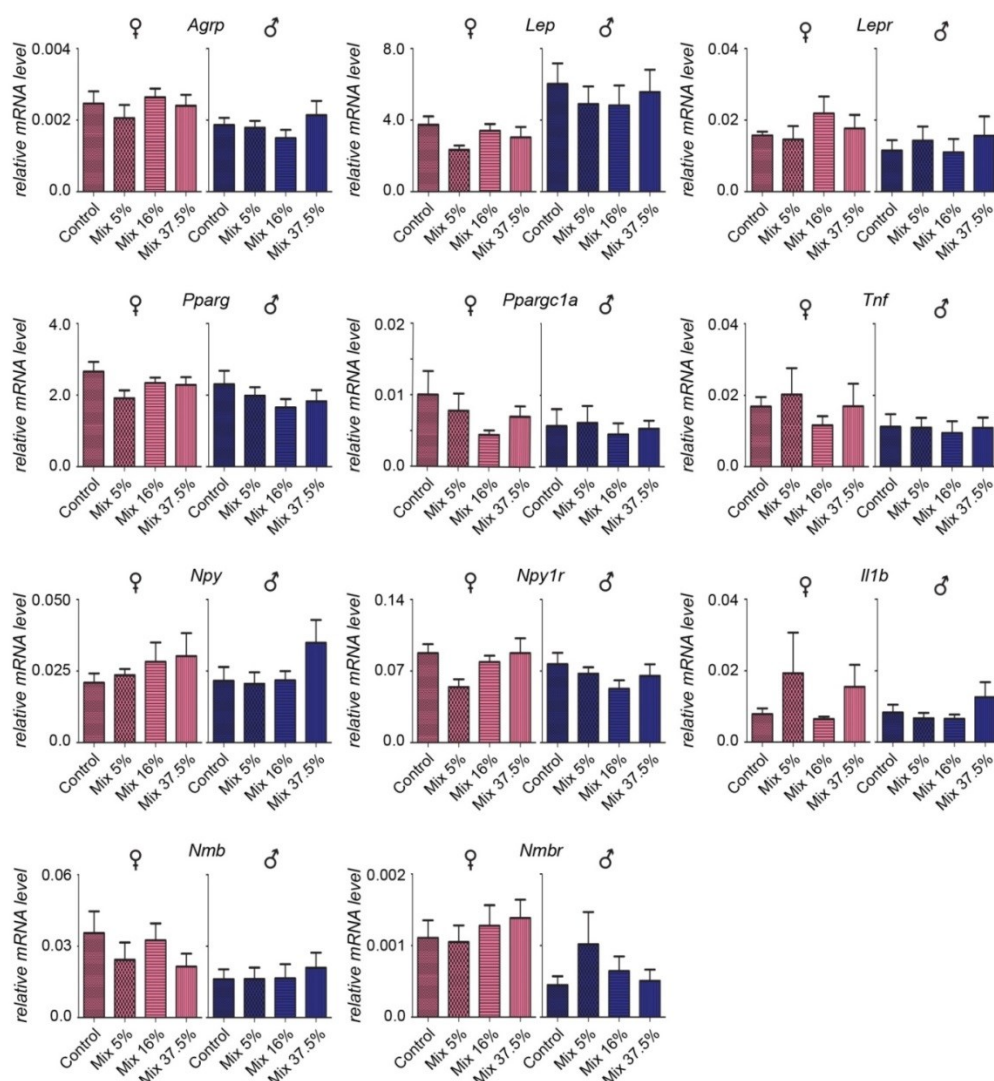


Figure 20. Relative mRNA expression of obesity-related genes in fat tissue. The targeted gene array was verified by TaqMan RT-qPCR assays on 11 genes across the sex-specific groups (n=8-9 from each group). Pink and blue bar represents female and male groups, respectively. Although some genes show a trend towards either up- or down-regulation, none were significantly altered relative to controls as determined by one-way ANOVA analysis. In control animals, *Nmb* was expressed significantly higher in females than in males ($p=0.030$), with *Nmb* appearing higher in females ($p=0.075$) and *Lep* appearing higher in males ($p=0.088$) as determined by two-tailed, unpaired t-test analyses.

Body weight, growth rate and food consumption in adult offspring

The body weight of the offspring was monitored approximately every 14 days from weaning until sacrifice (fig. 21). The weights were analysed over the time-span with a repeated measures ANOVA. The weight over time of the male offspring was reduced in all dose groups compared to control with $p=0.0172$, $p=0.0391$ and $p=0.0169$ for respectively Mix-5, Mix16 and Mix-37.5. In the females Mix-37.5 there was a tendency toward decreased body weight (LSMeans $p=0.06$ and Dunnett's adjusted $p=0.03$). Hence, despite there being no difference between groups among the weaned offspring, the male offspring from the dosed groups weighed less than the controls for the whole time-period after weaning.

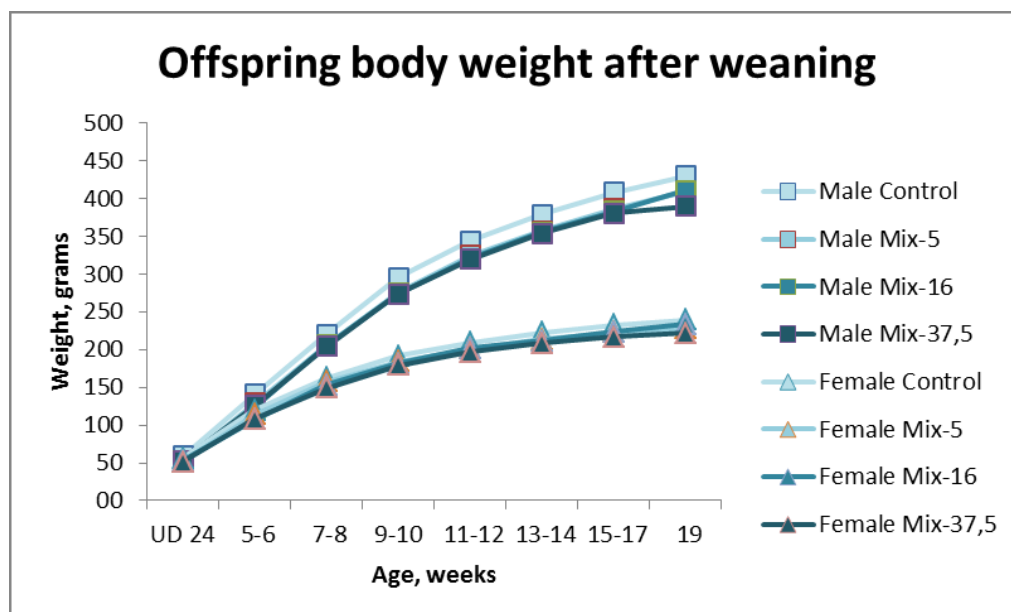


Fig. 21. Offspring body weight after weaning, male and female. Weights were obtained at different ages for the different blocks and grouped according to the age of the offspring for the purpose of depicting data. The statistical analysis was performed with the exact timing of the weight included. The weight in all exposed groups was statistically significantly reduced compared to controls. $n = 18-20$. Group means are depicted.

At 14 weeks of age the Specific growth rate (SGR) was determined for the offspring. There was no statistically significant difference between dose groups and control (figure 22). Food consumption and energy efficiency 14 weeks of age was assessed. A sex difference was observed for both parameters but no differences between the exposure groups were found (figure 23).

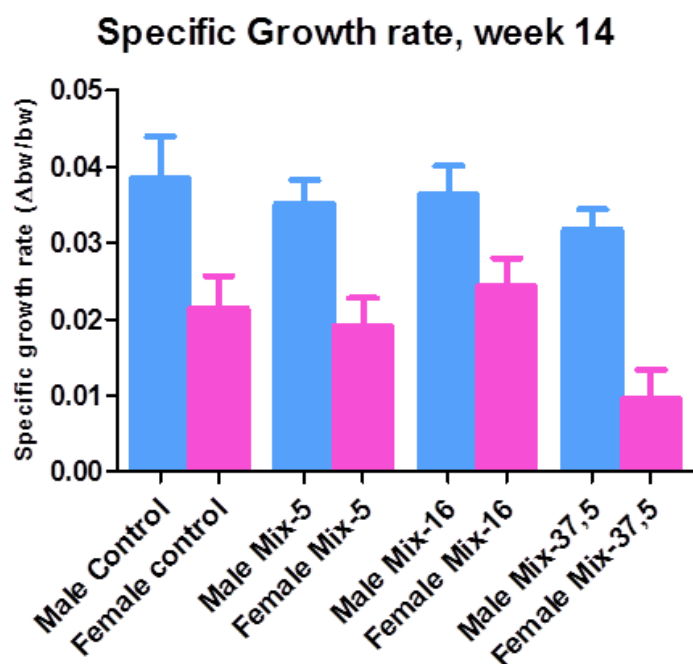


Fig 22. Specific growth rate in offspring 5 months of age. $n = 18-20$. Mean+SEM

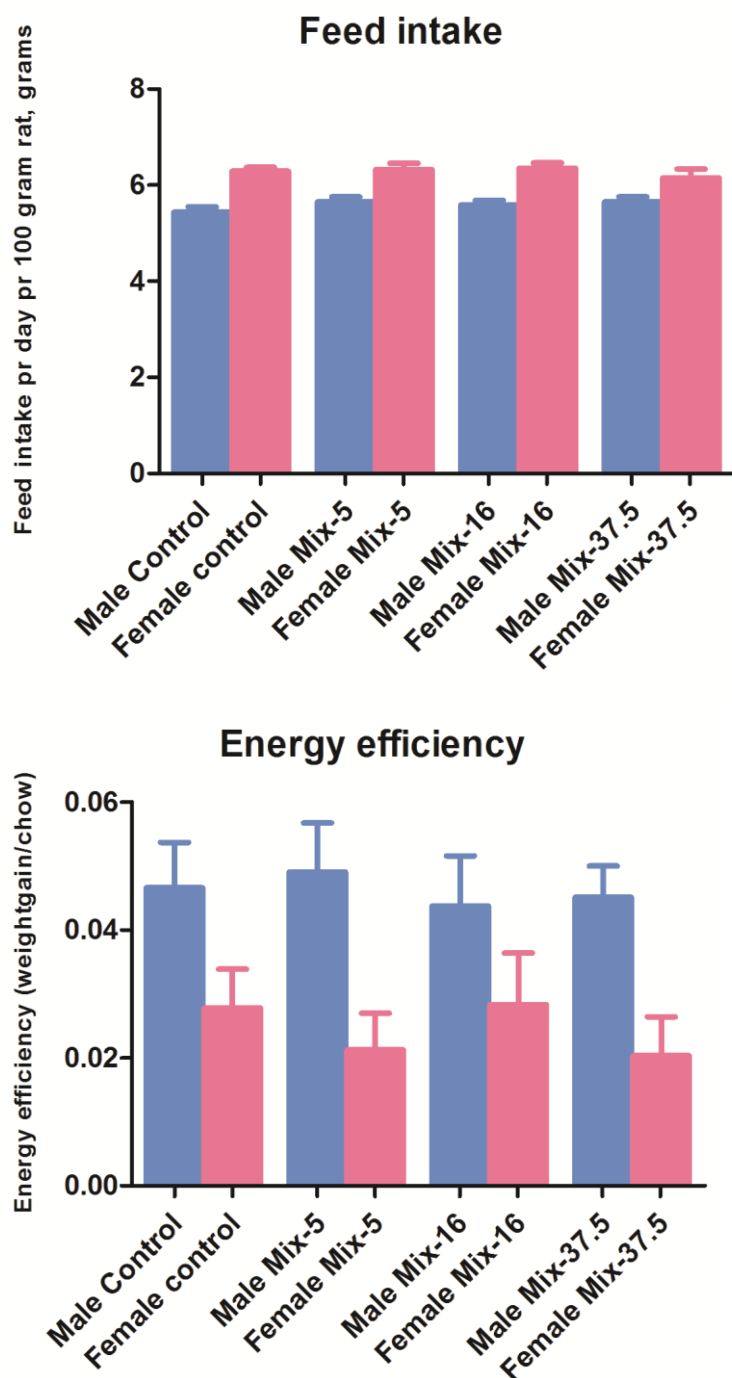
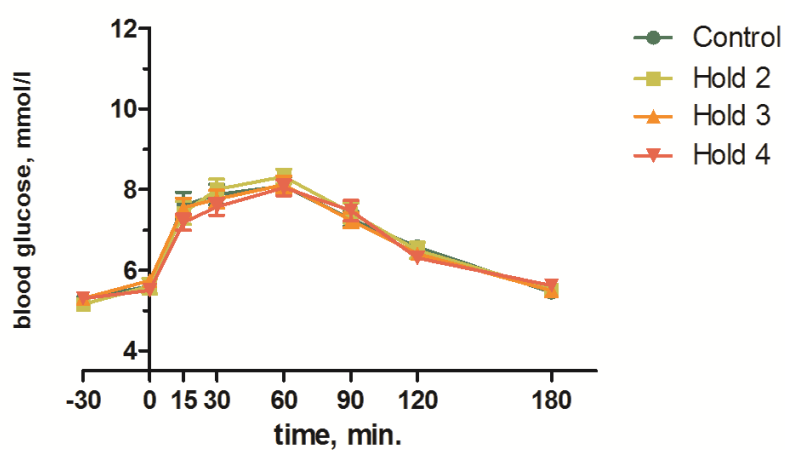


Fig. 23 Food intake and energy efficiency in offspring 14 weeks of age. n = 8-9. Mean+SEM

Glucose Tolerance test

When the offspring were 15 weeks old no dose-related effect on glucose tolerance was observed (figure 24). In connection with this testing fasting insulin and glucose was measured and no effect on these parameters was found. The insulin levels had a large variation leading to low sensitivity for detecting effect figure 25).

Oral glucose tolerance males 15 weeks of age



Oral glucose tolerance females 15 weeks of age

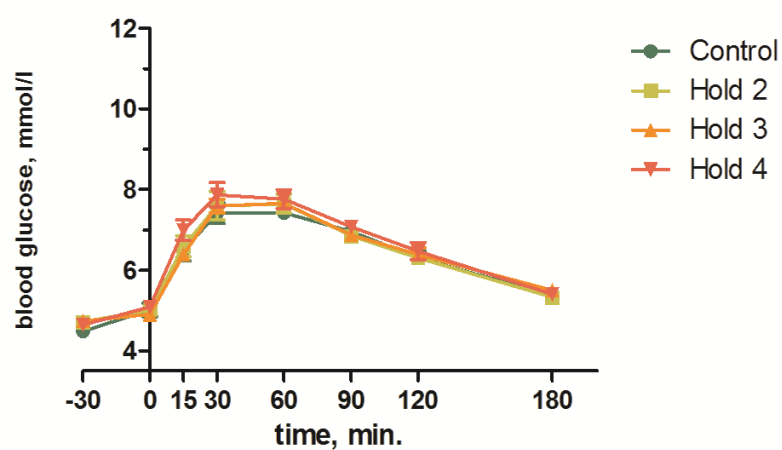


Fig. 24. Oral glucose tolerance test performed on offspring at age 15 weeks. Glucose administered at time 0. Mean \pm SEM

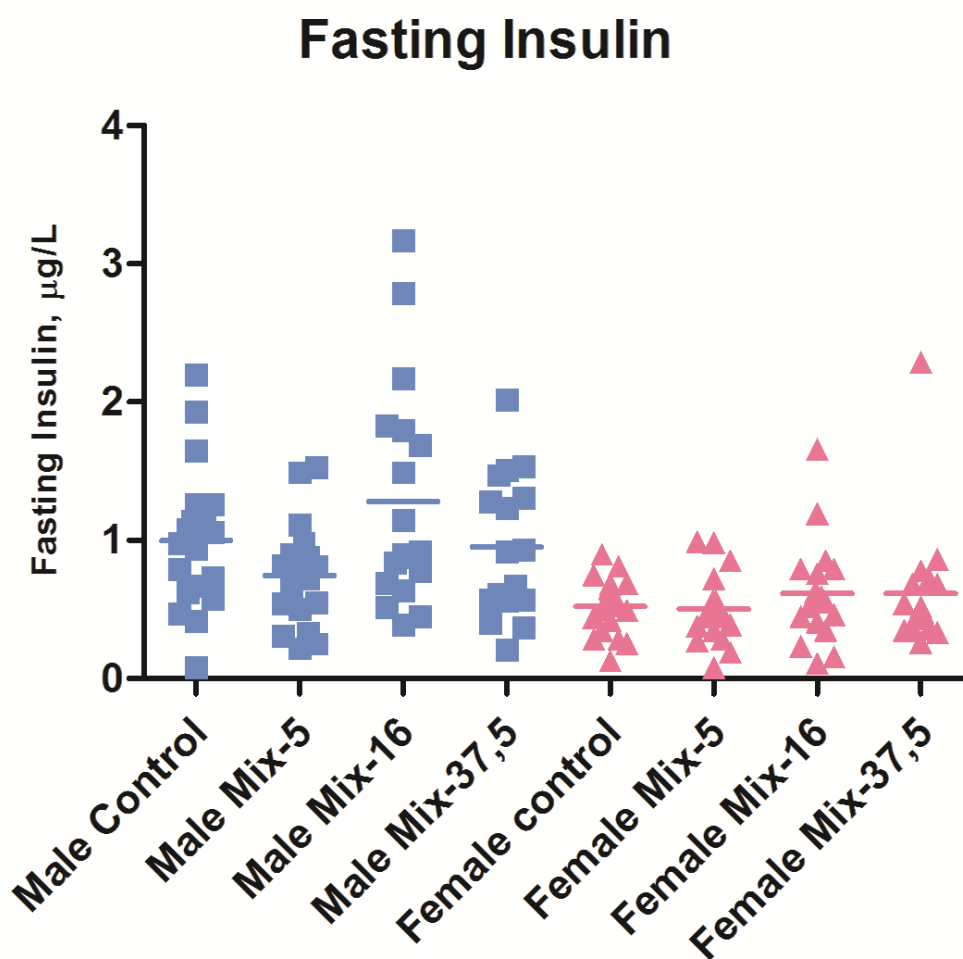


Fig. 25. Fasting insulin shown as group mean and data from individual animals.

3.3.4 Summary of findings

In the following the findings in the large dose-response study and the range-finding study are summarized and also compared in case the endpoints were assessed in both studies. The overall discussion of the main findings is in chapter 4.

Toxicity to dams and effects on pregnancy parameters

Maternal effects were seen in the groups exposed to Mix-16% and Mix-37.5% in the form of slightly decreased mean maternal weight gain. This replicates the finding from the range-finding study, where only a significant effect was seen in dams exposed to Mix-37.

The liver weight of the dams exposed to Mix-37.5% was increased in the range-finding study on PD 16 where the dams were still exposed. However, no effect on liver weight was seen at any of the doses, incl. Mix-37.5% in the main study on PD 24, i.e. 8 days after the end of exposure. Thus, this effect appeared to be reversible after cessation of the exposure. There were no effects on gestation length, litter size or pup survival. Based on the above, it is evaluated that Mix-16% and Mix-37.5% induced only slight maternal toxicity.

Mixture effect on birth weight and postnatal growth

The exposure to the mixture caused a dose-related decrease of the mean birth weight at all doses. The individual pesticides were present in the mixture at lower doses than those leading to decreased birth weight. Thus the effect of the mixture is a very clear sign of combination effects on birth weight at doses where individual pesticides do not show any effect. The predicted effect with the use of dose-addition corresponded well with the observed results. Decreased pup body weight was seen up to PD 24 at the highest dose of the mixture. The offspring exposed to the two lower doses appeared to show some catch-up on body weight.

Other developmental toxicity effects, incl. AGD, NR and mammary gland development

AGD was significantly decreased at all doses in both male and female pups in the range-finding study. The AGD in male offspring was not affected by the exposure in the main study where more litters per dose group were included. In female offspring, AGD was significantly longer at the highest dose level, i.e. Mix-37.5%. Nipple retention in the male pups appeared marginally higher than controls in the Mix-37.5% group and this was seen in both studies, however the difference was only statistically significant in the main study.

A decreased mammary outgrowth towards the lymph nodes in PD 24 males was found in the lowest dose-group although large variations were present for whole mount measurements. A non-significant trend to reduced area and reduced ductal elongation in the same dose-group supports the finding in the male offspring. This may indicate a non-monotonic dose-response on mammary outgrowth of the pesticide mixture.

Biomarkers for obesity

In the range-finding study a significant increase of glucagon plasma levels in male offspring UD16 was found in the Mix 37.5% dose group. The main study also appeared to show an increase relative to controls in the Mix 37.5% dose group, but the difference was not statistically significant. The main study included more animals per group than the range-finding study and is therefore considered to provide the most conclusive data. However, seen together the two studies indicate that there may be an effect on glucagon levels at high doses, but no strong conclusions can be drawn.

A significant increase in leptin was observed in the female offspring age 4-5 month in the highest dose group.

Gene expression

In our initial gene array, we observed a handful of genes that were dysregulated in adipose tissue (retroperitoneal fat pad) from young adult rats following intrauterine exposure to a mixture of pesticides. For instance the Neuromedin B receptor (Nmbr) was up-regulated in all dose groups, whereas its ligand, Neuromedin B (Nmb) was unaffected or down-regulated (in Mix-16% group). However, follow-up expression analyses on a larger biological sample size failed to show a significant alteration of Nmbr expression in the exposed animals. Interestingly, we saw a significant higher (2.5-fold) expression of Nmbr in female adipose tissue than in male.

Food consumption, energy efficiency and Oral glucose tolerance test

The food consumption, energy efficiency and oral glucose tolerance was assessed in the offspring at 14-15 weeks of age. No differences between groups were found.

Organ weights and histology of liver and pancreas

At the high dose, PD 16 offspring showed a significantly increased weight of fatty tissue relative to the body weight and a tendency was still seen in males in adulthood. Other organ weights were not dose-dependently affected in offspring at any of the time-points investigated, indicating no changes in the investigated organs involved in glucose metabolism (muscle and liver weights). This was further confirmed by histological evaluations of pancreas and liver. Morphometric evaluation of pancreas histology showed no effects on pancreatic islet size, number or distribution of β - and α -cells in offspring PD 16 or in adulthood. This is in good accordance with our findings on insulin and glucagon plasma levels, where no changes were found either. Although a pilot screen on gene expression levels of β -cells showed some apparent effects PD 16, it appears that pancreas was not affected by perinatal exposure to the mixture of pesticides either on PD 16 or later in adulthood.

The mixture did not appear to give rise to hepatotoxicity in offspring or 8 days post-exposure in dams based on liver weights. This was further confirmed by histologic evaluation of livers from offspring PD 16.

4. Discussion

4.1 Mixture effect on birth weight

This project generally aimed to explore the hypothesis that combined developmental exposure to pesticides causing decreased birth weight could lead to this effect at dose levels below NOAELs for the single pesticides, and to investigate if dose-additivity modelling of the expected mixture effects could give useful estimates of the observed mixture effects.

Mixture effect below NOAEL

Birth weight was affected by the combination of the six pesticides (Cyromazine, MCPB, Pirimicarb, Quinoclamine, Thiram, and Ziram) and the effect was seen at dose levels at which the individual pesticides were predicted to cause no, or only minor, effects on birth weight (Figure 26). To our knowledge, this is the first study to describe mixture effects on birth weight.

Mixture effects on reproductive development have been seen in studies where experimental animals have been exposed to several endocrine disruptors simultaneously even though each of the individual substances were present at low, non-adverse doses (Hass et al 2007, Christensen et al 2009, Hass et al. 2012a,b). We have earlier shown that a mixture of the fungicides procymidone, mancozeb, epoxyconazole, tebuconazole and prochloraz induced severe effects on reproductive development manifested as nipple retention and genital malformations in male offspring at doses where the individual pesticides appear to induce no such effects. These mixture studies of endocrine disruptors have also included substances with dissimilar mode of action e.g. AR-antagonists and substances leading to decreased testosterone levels. However, they all lead to disruption of sex hormone functions. Based on such data and others, a state of the art science document on mixture has proposed a science-based approach for performing cumulative risk assessment of pesticides with dissimilar modes of action (Kortenkamp et al. 2012). It is recommended to use a tiered framework analysis also for the assessment of mixtures of dissimilarly acting chemicals (Kortenkamp et al. 2012). At lower tiers of the analysis, all chemicals should be assessed, irrespective of their presumed modes of action. At higher tiers, chemicals known not to contribute to a relevant common adverse outcome can be excluded from the analysis. By way of further refining the analysis, criteria for the grouping of chemicals into common assessment groups based on common adverse endpoint, should be applied.

According to the above recommendations, all substances known to cause reproductive and developmental toxicity could be considered together at lower tiers. However, reproductive toxicity comprises a broad range of different effects, ranging from reductions in fertility to developmental toxicity effects. At higher tiers, such diverse effects may have to be differentiated. For developmental toxicity effects, adverse outcomes are often differentiated into structural abnormalities, functional deficiencies, death of the developing organism and altered growth (ECHA 2009). Altered growth may then be further differentiated into increased or decreased growth, where decreased birth weight constitutes an important endpoint for decreased in utero growth. The present results showing clear mixture effects of pesticides causing decreased birth weight at low, non-adverse doses support the relevance of performing cumulative risk assessment for this type of effect.

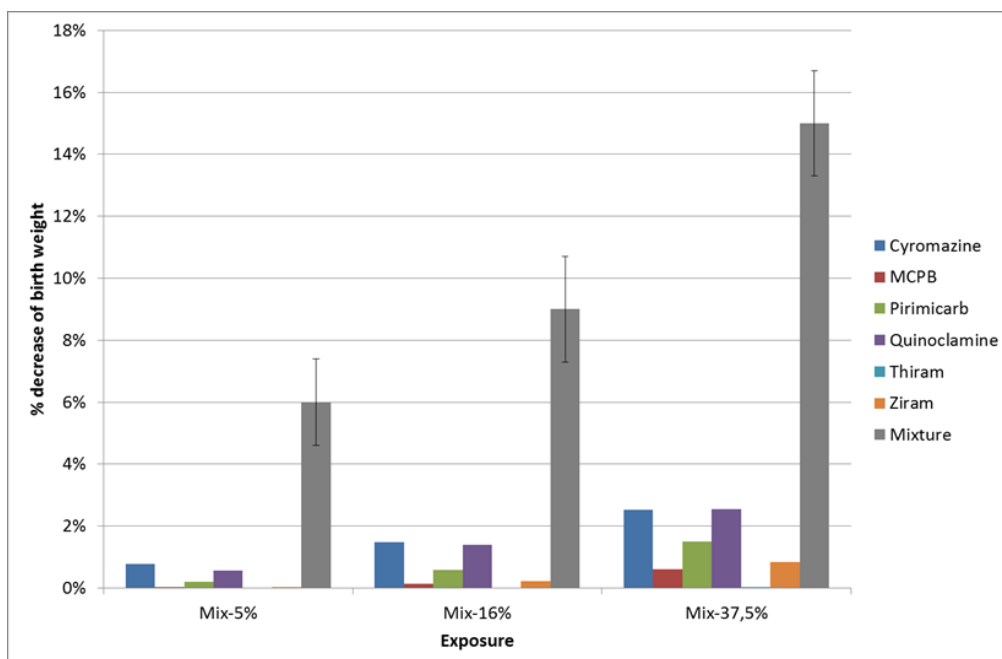


Figure 26 Decreased birth weight (%) after exposure to the pesticide mixture (observed results) and the individual pesticides at the doses included in the mixture (predicted effect).

Prediction of mixture effect on birth weight

Decreased birth weight may be induced via many different and in most cases unknown mechanisms of action. Thus, independent action (IA) may provide a more accurate prediction of mixture effect on birth weight than dose addition (DA). The state of the art document on mixtures states that there is no current example of a situation in which the concept of IA provides an accurate prediction that is also more conservative (i.e. cautious) than DA, supporting the use of DA as a conservative default in cumulative risk assessment (Kortenkamp et al 2012). Also, an analysis of the quantitative difference between predictions based on DA or IA suggested that the differences that might be expected in practice are small. Our results have shown that application of the DA and IA model lead to almost similar predictions and resulted in good predictions of the observed mixture effects. Therefore, the results in the present project support the use of DA for cumulative risk assessment for the endpoint decreased birth weight.

Regulatory perspectives with regards to mixture effect on birth weight

In the present project mixture effects of pesticides causing decreased birth weight have been clearly shown, and as such the results support that there is a need for risk assessment of the cumulative exposure to chemicals.

Below the mixture results are discussed in relation to values used in regulatory risk assessment of single chemicals such as LOAELs and NOAELs (See sections 2.2 and 2.3 for details and references).

The observed LOAELs for the pesticides individually and the doses of the pesticides in the mixture at the mixture LOAEL were compared (Table 18). The main purpose was to estimate whether mixture exposure would lead to lower LOAELs for effect on birth weight than seen after single chemical exposure. This was clearly the case as the doses of the single pesticides at the mixture LOAEL were approximately 20-40 times lower than the LOAELs for effect on birth weight when the pesticides were given alone. Also, the doses of the single pesticides at the mixture LOAEL were also lower (approximately 5-17 times) than the NOAELs found for the pesticides when given alone. The observed LOAELs and NOAELs for the effect on birth weight after exposure to the single pesticides depend very much on how the doses were selected in the studies and therefore a comparison of the estimated ED5 for each pesticide with the doses of the single pesticides at the mixture LOAEL was also done. This showed that the mixture LOAEL

was 20 times lower than the estimated ED₅'s for the pesticides when given alone. Overall, this illustrates that mixture exposure leads to markedly lower effect levels for individual pesticides within the mixture than those seen after exposure to single substances. Also, it shows that mixture effects occur at dose levels where the single substances show no effect on birth weight, i.e. at the observed NOAELs for this effect. This finding is supported by earlier results from us and others (Hass et al. 2007, Christiansen et al. 2009, Hass et al. 2012).

Table 18 Effect on birth weight. LOAELs, NOAELs and ED₅s (mg/kg bw/day) for effect on birth weight or foetal weight GD 21 after developmental exposure to the single pesticides alone (suffix P) and the dose of the single pesticides at the LOAEL for the mixture (LOAEL_{mix}).

| Pesticide | LOAEL _P | NOAEL _P | ED _{5P} | LOAEL _{mix} , mix-5% | LOAEL _P /LOAEL _{mix} | ED _{5P} / LOAEL _{mix} | NOAEL _P / LOAEL _{mix} |
|--------------|--------------------|--------------------|------------------|----------------------------------|---|--|--|
| Cyromazine | 600 | 300 | 350 | 17.5 | 34 | 20 | 17.1 |
| MCPB | 100 | 25 | 100 | 5 | 20 | 20 | 5.0 |
| Pirimicarb | 75 | 25 | 60 | 3 | 25 | 20 | 8.3 |
| Quinoclamine | 20 | 5 | 10 | 0.5 | 40 | 20 | 10.0 |
| Thiram | 30 | 16 | 20 | 1 | 30 | 20 | 16.0 |
| Ziram | 25 | 12.5 | 20 | 1 | 25 | 20 | 12.5 |

It has been argued that mixture effects are not seen at or below the NOAELs used for deriving e.g. ADI for single pesticides (COT 2002, VKM 2008). This could be the case if other effects occurring at lower doses have been used for deriving the ADI than those effects studied in a mixture study. As our mixture was not based on the NOAELs used for deriving the ADI, but on ED₅ for effect on birth weight, we cannot directly address this question based on our data. However, Table 19 shows a comparison of the NOAELs used for deriving the ADI (mg/kg bw/day) for the single pesticides alone (suffix ADI) and the dose of the single pesticides at the LOAEL for the mixture (suffix mix). For one of the pesticides (Quinoclamine), the dose at the LOAEL for the mixture is clearly lower than the NOAEL used for deriving the ADI, i.e. around 6 times lower. For pirimicarb and MCPB, the doses in the mixture are slightly lower (1.2 or 1.3 times) and for cyromazine, thiram and ziram the LOAEL when given in the mixture is 3-10 times higher than the NOAEL used for deriving the ADI. These data therefore does not give a clear answer. However, it should be kept in mind that our study did not find a mixture NOAEL for the effect on birth weight which means that the mixture LOAEL might be lower than found in our studies. Last, but certainly not least our mixture included only 6 pesticides among the 175 pesticides that may induce decreased birth weight. Therefore, the present data can most certainly not be used to conclude that mixture effects on birth weight will not occur at the NOAELs used for deriving ADIs for pesticides.

Table 19 Effect on birth weight. The NOAELs used for deriving the ADI (mg/kg bw/day) for the single pesticides alone (suffix ADI, data from EFSA website) and the dose of the single pesticides at the LOAEL for the mixture (LOAEL_{mix}).

| Pesticide | Effect of single pesticides and the NO-AEL used for deriving the ADI | NOAEL _{ADI} | LOAEL _{mix} : mix-5% | NOAEL _{ADI} /LOAEL _{mix} |
|--------------|--|----------------------|-------------------------------|--|
| Cyromazine | A semi-chronic study in dogs supported by a long-term study in mice leading to a NO-AEL of 6 mg/kg. The critical effects include decreased body weight gain, liver weight changes and haematological and clinical chemistry changes in dogs and decreased body weight gain in mice. ADI is 0,06 mg/kg bw/day | 6 | 17.5 | 0.3 |
| MCPB | A Repeated Dose 90-Day Oral Toxicity Study in Rats leading to a NOAEL of 100 ppm (6.3mg/kg bw/d). The critical effects include kidney, haematology. ADI is 0.01 or 0.063 mg/kg bw/day. | 6.3 | 5 | 1.3 |
| Pirimicarb | NOAEL of 3.5 mg/kg bw/day from 1-yr dog study, ADI is 0,035 mg/kg bw/day | 3.5 | 3 | 1.2 |
| Quinoclamine | The NOAEL is 3 mg/kg bw/day based on increased relative liver weight, blood parameters and kidney pigment formation at 10 mg/kg bw/day. | 3 | 0.5 | 6.0 |
| Thiram | NOAEL of 0.84 and 1.5 mg/kg bw/d (lowest NOAEL), identified in a 12 month chronic feeding study in the dog and 2 year, reproductive toxicity study in rats. ADI is 0.01 mg/kg bw/d | 0.1 | 1 | 0.1 |
| Ziram | Long-term toxicity in rats or mice, the LOAEL being 2.5 and 3 mg/kg bw/day respectively. ADI is 0.003 mg/kg bw/day | 0.3 | 1 | 0.3 |

Mixture effect on the dams (maternal toxicity)

Maternal toxicity in the form of slightly reduced maternal weight was seen at a higher mixture dose i.e. the middle dose (Mix-16%), than the dose inducing decreased birth weight, i.e. the lowest mixture dose (Mix-5%).

Table 20 shows the reported LOAELs and NOAELs for effects on the dams for the pesticides singly and the doses of the pesticides in the mixture at the mixture LOAEL where we observed effect on the dams. The main purpose was to estimate whether mixture exposure would lead to lower LOAELs for effect on the dams than seen after single pesticide exposure. This was clearly the case as the doses of the single pesticides at the mixture LOAEL were approximately 5-11 times lower than the LOAELs when the pesticides were given alone. Also, the doses of the single pesticides at the mixture LOAEL were lower (approximately 1.4-7 times) than the NOAELs found for the pesticides when given alone. This illustrates that also for effects on the dams mixed exposure leads to lower effect levels than those seen after exposure to single substances. Also, it shows that mixture effects occur at dose levels where the single substances show no effect on dams, i.e. at the observed NOAELs for this effect.

Table 20 Effect on the dams. LOAELs and NOAELs after exposure during gestation to the single pesticides alone (suffix P) and the dose of the single pesticides at the LOAEL for the mixture (LOAEL_{mix}).

| Pesticide | Study design | LOAEL _P | NOAEL _P | LOAEL _{mix} : mix-16% | LOAEL _P / LOAEL _{mix} | NOAEL _P / LOAEL _{mix} |
|--------------|--|--------------------|--------------------|-----------------------------------|--|--|
| Cyromazine | Reduced bw, prenatal dev. tox study | 300 | 100 | 64.8 | 5 | 1.5 |
| MCPB | Prenatal dev. tox study | 100 | 25 | 18.5 | 5 | 1.4 |
| Pirimicarb | Prenatal dev. tox study | 75 | 25 | 11.1 | 7 | 2.3 |
| Quinoclamine | Prenatal dev. tox study; LOAEL~20-75, NOAEL ~ 5-100; 4 studies with somewhat inconsistent data, hunched posture at 37.7, enlarged spleen at 20 | 20 | 5 | 1.9 | 11 | 2.6 |
| Thiram | Prenatal or perinatal study; NOAEL not found | 17 | - | 3.7 | 5 | >5 |
| Ziram | Prenatal dev. tox study, NOAEL not found | 25 | - | 3.7 | 7 | >7 |

That mixture effects occur at dose levels where the single pesticides show no effect on dams, i.e. at the observed NOAELs for this effect, is supported by earlier mixture results from us in a project where the mixture ratio was chosen according to the doses of each individual pesticide that produced no observable effects on the dams incl. pregnancy length and pup survival in our laboratory. The doses studied ranged from 25 to 100% of this mixture. The range-finding results showed clearly that a mixture of the fungicides procymidone, mancozeb, epoxiconazole, tebuconazole and prochloraz induced severe effects in the dams manifested as dystocia (impaired parturition) and increased gestation length at doses where the individual pesticides appear to induce no such effects (Jacobsen et al. 2010).

Similar results were found in the main study as increased gestation length was found at mixture doses where the individual pesticides induced no such effect (Hass et al. 2012). Application of the dose-addition model resulted in a good prediction of the observed mixture effects.

The significance of these findings for human risk assessment must be emphasised, because they clearly indicate that also for pregnant animals risk assessment based on single substances alone underestimates the risk for adverse effects when exposure is to several pesticides with common effect outcomes regardless of mechanism.

4.2 Metabolic syndrome

The Barker theory, based on human studies, describes an association between low birth weight and coronary disease later in life (de Boo and Harding, 2006). Further human studies have shown associations between low birth weight and insulin resistance and diabetes type 2 but also that poor growth during the first year of life succeeded by rapid growth in childhood may aggravate the risk for developing coronary heart disease, hypertension and diabetes as adults (reviewed by de Boo and Harding, 2006). *In vivo* studies on restricted protein intake during gestation in rodents, guinea pigs and sheep showed low birth weight and increased blood pressure and altered glucose tolerance in the offspring, indicating a possible link between low birth weight and metabolic syndrome (reviewed by de Boo and Harding, 2006).

As our range-finding study showed low birth weight after gestational and lactational exposure to a mixture of pesticides, it was relevant to further investigate whether the offspring would develop signs of metabolic syndrome in adulthood. We focused on metabolic biomarkers for obesity and for diabetes, organ weights related to obesity and diabetes, effects on pancreas (gene expression and histology) and insulin resistance.

Body weights, growth, food consumption, oral glucose tolerance test in offspring

In the main study, the birth weight of the offspring was reduced in all dose groups and both sexes compared to controls. The decreased body weight in the adult Mix-37.5 males and females indicate the opposite of catch-up growth. Thus, the study shows no indications of catch-up growth and obesity due to low birth weight.

There was no effect on specific growth rates (SGR), food consumption, energy efficiency and oral glucose tolerance in any of the sexes or dosed groups.

The oral glucose tolerance test offers a dynamic picture of the glucose handling in response to an oral glucose load. By measuring the serum glucose concentrations at different time points it was seen that the glucose tolerance of all animals was similar at the tested age. Impaired glucose tolerance would have manifested as elevated glucose levels in the final part of the test. However, due to the low glucose peak in this test (only around 8 mmol/l) it is possible that a potential difference between the groups due to a modest impact on the system could not be revealed. The calculated HOMA-IR score does not imply any differences between groups as all animals had similar insulin resistance according to this model. It is possible that a commencing insulin resistance could have been manifested during the glucose test as elevated insulin levels maintaining the same serum glucose in all groups. In this experiment it was not possible to include these dynamic measures of insulin resistance. There are indications that effects in the animals on these endpoints related to the development of Diabetes type 2 may not manifest themselves until the animals are aging. In other studies, protein restriction during foetal life caused an earlier age-related pre-diabetic state than in control rats but the animals had normal glucose handling during adult life (Hales et al., 1996, Portha et al. 2011). Hence, the present lack of effects at the age of 4-5 months does not rule out that effects would be seen later in life, e.g. in offspring between 1 and 1.5 years old.

Organ weights and histology of liver and pancreas

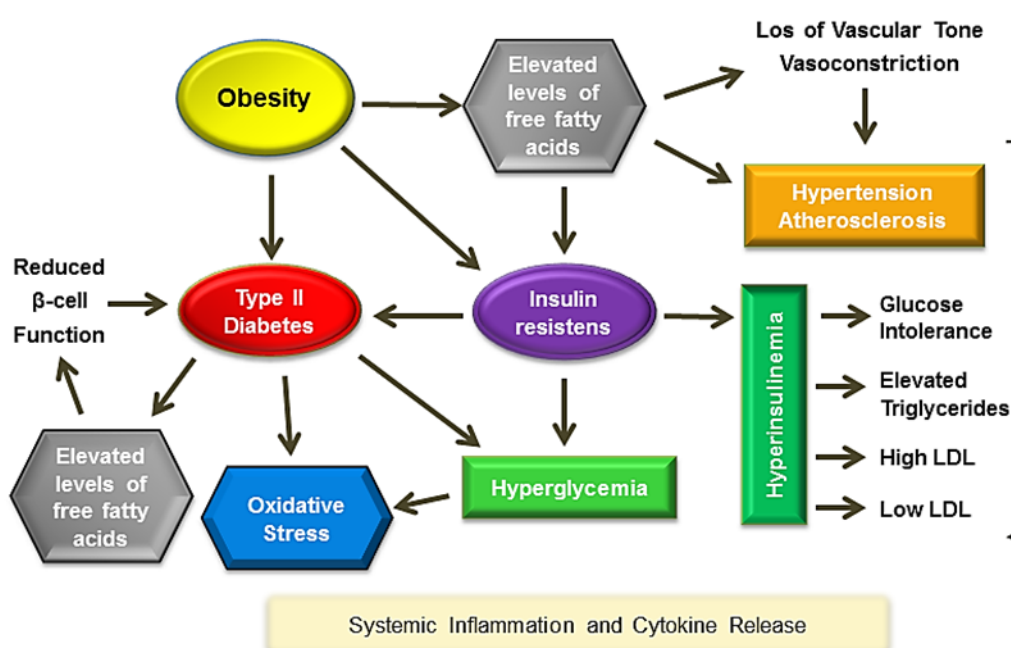
At the high dose, PD 16 offspring showed a significantly increased weight of fatty tissue relative to the body weight and a tendency was still seen in males in adulthood. Other organ weights were not dose-dependently affected in offspring at any of the time-points investigated, indicating no changes in the investigated organs involved in glucose metabolism (muscle and liver weights). This was further confirmed by histological evaluations of pancreas and liver. Morphometric evaluation of pancreas histology showed no effects on pancreatic islet size, number or distribution of β - and α -cells in offspring PD 16 or in adulthood. This is in good accordance with our findings on insulin and glucagon plasma levels (see below), where no clear changes were found either. Although a pilot screen on gene expression levels of β -cells (see below) showed some apparent effects PD 16, it appears that pancreas was not affected by perinatal exposure to the mixture of pesticides either on PD 16 or later in adulthood.

The mixture did not appear to give rise to hepatotoxicity in offspring or 8 days post-exposure in dams based on liver weights. This was further confirmed by histologic evaluation of livers from offspring PD 16.

Biomarkers for obesity

Obesity is associated with increased risk for diabetes, hypertension, atherosclerosis and metabolic syndrome. The complexity of these pathologies has driven an increased demand for quantitative measurement of biomarkers linked to these disease states.

Morbid obesity is characterized by a chronic inflammatory state, resulting in an array of chronically dysregulated inflammatory biomarkers secondary to altered endocrine function of the adipocytes (fig 27). Typically pro-inflammatory cytokines and chemokines, such as TNF- α , and IL-6 are up regulated in obesity, whereas other anti-inflammatory mediators, such as adiponectin are typically down regulated. Obesity is also characterized by increased insulin resistance, and obese people often will express increased levels of insulin, secondary to insulin resistance, and insulin-like growth factor-1 (IGF- 1).



Ref.: Development of Biomarker Assays for Obesity, Diabetes, and Metabolic Syndrome. Bruk G. Leta, Steven C. Hof Selen A. Atromgren, Pankaj Oberoi, Eli Glezer, Robert m: Umek, and Jacob N. Wohlstadter. Meso Scale Discovery A

Figure 27 Morbid obesity and systemic inflammation resulting in an array of chronically dysregulated inflammatory biomarkers (see reference in the figure)

In our study on rats, the investigation of the effect of pesticide exposure on biomarkers related to metabolic syndrome, obesity and diabetes showed no clear effects on the plasma levels of the measured hormones, except for a significant increase in plasma leptin levels in female offspring age 5-6 months exposed to the highest mixture dose (mix-37.5 %). Leptin is one of the cytokines produced in fat tissue. It is secreted in direct proportion to adipose tissue mass and it is one of central regulators of energy homeostasis (Vickers, 2007). We have previously shown that exposure to butylparaben and diisobutylphthalate during development reduced leptin and insulin levels in rat foetuses (Boberg et al. 2008). Interestingly, a study in European adolescents has tested the hypothesis that a lower birth weight, as an indicator of an adverse intrauterine environment, is associated with higher leptin levels (Labayen et al. 2011). The results showed a sex-specific programming effect of birth weight on serum leptin levels, i.e. leptin levels were increased only in female adolescents.

Many forms of obesity are characterized by an increased level of circulating leptin. However, it has been shown that many obese individuals are resistant towards leptin. This indicates that leptin is mostly a marker of nutrition and not a satiety hormone as first believed, since it is unable to decrease weight in obese, and the level of leptin itself decreases with calorie restriction and weight loss (Kershaw et al., 2004).

A tendency towards dose dependent increase in C-peptide levels was also seen in the female offspring age 5-6 month. C-peptide is generally found in amounts equal to insulin because insulin and C-peptide are linked when first made by the pancreas. The level of C-peptide in the blood can be used as a measure of how much insulin is being made by the pancreas, but in contrast to insulin, C-peptide does not affect the blood sugar level in the body (Abdullah et al., 2012). In our study there was no effect (and no tendency to an increase) on the plasma insulin levels, so even though the observed increase in C-peptide seemed to be dose dependent it could just be a change finding, and the effect was not statistically significant.

The significant increase in glucagon in male offspring UD16 from the Mix 40% dose group seen in the range finding study was not seen in the main study, although there was a tendency towards a slight increase in the 37.5% dose group in the main study.

Glucagon is produced by alpha cells of the pancreas, and raises the concentration of glucose in the bloodstream, the opposite effect of insulin, which lowers the glucose concentration. The pancreas releases glucagon when the concentration of glucose in the bloodstream falls too low. Glucagon causes the liver to convert stored glycogen into glucose, which is released into the bloodstream, and high blood glucose levels stimulate the release of insulin (Reece et al., 2002). Thus, glucagon and insulin are part of a feedback system that keeps blood glucose levels at a stable level. Therefore in that respect the lack of effect on glucagon levels fits together with there being no effect on the measured insulin and glucose levels, in the present study.

Overall, studies on biomarkers of obesity are unfortunately still producing non-convincing results. For instance, adiponectin has been inversely correlated to the risk of cardiovascular diseases in men, but not in women. Leptin, a direct modulator of pancreatic β -cell function, has been shown to be an independent predictor for development of diabetes type 2 in men, but not in women. In other studies, leptin has been shown to be a predictor of cardiovascular diseases, but opposite results have also been reported (Roos et al., 2012).

In addition previous studies on prenatal exposure to environmental chemicals and effects on obesity or obesity related endpoints, have shown that some effects first appear later in life i.e. in adulthood or senescence. Therefore it is possible that the lack of effects on many of the investigated biomarkers is because the effects are not evident at the ages investigated (young 4-5 months old animals and UD 16 old males), but appears later in life. Also human studies describes an association between low birth weight and increased risk of obesity later in life (Schultz et al. 2014; Jornayvaz et al. 2016), but results from this study on offspring kept into adulthood shows no indication of catch-up growth and development of obesity or metabolic syndrome, though a dose-dependent decrease in body weight of male and female offspring was observed. Therefore the overall lack of effects on the measured biomarkers can also be because the animals are in fact not on the way to become obese.

Gene expression

An animal's decision to eat or abstain is based on interpretations of a complex network of biomolecular signalling events involving several organs and tissues, including fat. Apart from storing energy in the form of lipids, the adipose tissue also functions as an endocrine tissue involved in the regulation of appetite, metabolism and the hypothalamic-pituitary-gonadal (HPG)-axis (Kawwass et al, 2015; Waki & Tontonoz, 2007). It produces and receives a large number of molecular factors, not least adipokines, but also growth factors, prostaglandins, sex steroids and more (Trayhum & Wood, 2004; Waki & Tontonoz, 2007). Therefore, disruptions to such factors can affect both the development and homeostasis of the adipose tissue. But alternatively, dysregulated factors can also function as useful biomarkers for metabolic syndrome or adipocyte-related functions.

In our initial gene array, we observed a handful of genes that were dysregulated in adipose tissue from young adult rats following intrauterine exposure to a mixture of pesticides. For instance the Neuromedin B receptor (*Nmbr*) was up-regulated in all dose groups, whereas its ligand, Neuromedin B (*Nmb*) was unaffected or down-regulated (in Mix-16% group). NMB is expressed in many organs and is involved in energy balance regulation (Hoggard et al, 2007; Moody & Merali, 2004). NMBR is also broadly expressed, but receptor function appears at least partially redundant as female knockout (KO) mice did not present with any severe phenotypes (Paula et al., 2010). However, female *Nmbr*-KO mice were partially resistant to diet-induced obesity. Interestingly, although follow-up expression analyses on a larger biological sample size failed to show a significant alteration in *Nmbr* expression in the exposed animals, we saw a significant higher (2.5-fold) expression of *Nmbr* in female adipose tissue than in male. Unfortunately, Paula et al. (2010) only analysed female KO mice, whereas it would be potentially insightful to see if there were other effects on male *Nmbr*-KO mice.

The fact that we observed significant differences in relative transcript abundance of certain genes between male and female retroperitoneal fat tissue is of great interest. Not only does it point towards fat tissue being sexually dimorphic at the molecular level, but also highlights the need to consider this sexual divergence when assessing any parameters potentially involving adipocyte tissues, being it centrally or peripherally. In fact, we also observed a higher expression level of Leptin (*Lep*) in male adipose tissue than in female of around 1.6-fold. Although not statistically significant, the trend was also persistent across all exposure groups. Thus, given the central role the leptin hormone plays in controlling food intake and energy expenditure (Münzberg & Morrison, 2015), this could have significant impact on how we study (and treat) obesity-related parameters unless sex of the subjects are accounted for.

Although some weak trends of dysregulation were discernible for the remaining genes, none showed significant changes in the exposed animals versus controls. For instance, the key regulator of metabolism and adipocyte differentiation, *Pparg* (Gregoire et al, 1998) appeared slightly downregulated in exposed groups, but inconclusively so. Also, the orexigenic hormone encoding gene *Npy* (Park et al, 2014) showed signs of upregulation in the Mix-37.5% groups of both sexes, but again not significantly. Thus, the gene expression data show indications of being disturbed following pesticide exposure, but further experiments are needed to clarify if these effects are significant or not. Being that most of these factors are relative broadly expressed throughout the body and often involved in paracrine signalling, additional studies at the protein levels could be of great value, also in adipose tissue.

4.3 Effects on sexual development

The range-find study showed significantly decreased AGD in male offspring at all doses. Decreased AGD in male offspring has been shown to be a sensitive endpoint for anti-androgenic effect and assessment of this effect was therefore also included in the main study. Here, no significant effect was seen, although the highest dose in the main study (mix-37.5%) was similar to one of the doses causing effect in the range-finding study. A comparison of the results in the two control groups and the two groups exposed to mix-37% is shown in table 21. The AGD results in the group exposed to mix-37% appear quite similar, whereas the control AGD is longer in the range-finding study than in the main study. This difference cannot be explained by the size of the male pups as the mean body weight appears higher in the main study than in the pilot study. Our historical control values for male AGD in 22 studies range from 20.5-24.6, so the control value in the range-finding study is clearly in the high end. This can happen especially in studies with a limited number of litters as in the range-finding study. Thus the significant effect of the pesticide mixture on male AGD found in the range-finding study is most likely not due to the exposure, but to an unusual control value. Also, the main study included more litters per group than the range-finding study (18-20 compared to 6-9) and therefore provides the most valid data. Thus, it is evaluated that no clear sign of effect was seen on male AGD.

Table 21. Comparison of AGD data from male offspring in the range-finding and the main study. * significantly lower than the control (p<0.05)

| Study/ group | Control | Mix-37% |
|--------------------------------------|------------|-------------|
| Range-finding, 6-9 litters per group | 24.4 ± 0.9 | 22.1 ± 0.6* |
| Main study, 18-20 litters per group | 21.7 ± 1.4 | 22.4 ± 1.2 |
| Study/ group | Control | Mix-37% |

In female offspring exposed to mix- 37.5% AGD was decreased in the range-finding study, but increased in the main study. Thus, these data are not consistent and taken together indicate that female AGD was unaffected.

Nipple retention was increased at 37.5% in the range-finding study (not statistically significant) and significantly increased in the main study. In both studies, the increases were small with only a few offspring showing several nipples. These results indicate anti-androgenic effect at the highest dose level of the mixture.

Reproductive organ weights may also be affected by exposure to endocrine disrupters, but our study did not find any effects on reproductive organs weight, incl. prostate/seminal vesicles and ventral prostate at the age of 4-5 months.

A decreased mammary outgrowth towards the lymph nodes in males was found in the lowest dose-group although large variations were present for whole mount measurements. A non-significant trend to reduced area and reduced ductal elongation in the same dose-group supports the finding in the male offspring. This may indicate a non-monotonic dose-response on mammary outgrowth of the pesticide mixture, however as the trend is only a trend and not significant this needs to be examined further to elucidate whether it is a true finding. Non-monotonous dose-response curves on mammary development have been reported for bisphenol A (Murray et al. 2007; Vandenberg et al. 2013), but showing the opposite effects, i.e. increased outgrowth as indications of an estrogenic mode of action. No effect was seen in female offspring, but gender specific sensitivity to disruption of mammary development has been reported before (You et al. 2002; Mandrup et al. 2012). It is therefore not clear whether the decreased mammary outgrowth reflects a true effect of the mixture on male mammary gland development.

The 6 pesticides included in the pesticide mixture were evaluated in the EFSA report Identification of Cumulative Assessment Groups of Pesticides (<http://www.efsa.europa.eu/en/supporting/doc/269e.pdf>). Three of the pesticides (MCPB, quinclamine and thiram) are placed in a common assessment group for anti-androgen effect based on in vivo findings including decreased testes and prostate weight, histological changes of testes and inhibition of spermatogenesis. Also, one of the other pesticides in the mixture (pirimicarb) is shown to be an aromatase inducer in vitro. Therefore, it seemed possible that the pesticide mixture could induce to endocrine disrupting effects via anti-androgenic or oestrogenic mode of action. The increased nipple retention in male offspring at the highest dose indicates anti-androgenic effect of the mixture. However, the small magnitude of this effect taken together with the lack of effect on AGD and reproductive organ weights, indicate that marked anti-androgenic effects may only be seen at very high doses of this specific pesticide mixture. The mixture induced slight maternal toxicity at the highest dose in the main study and severe maternal toxicity at an only two times higher dose in the range-finding study. It is therefore most likely not possible to investigate, if there would be marked anti-androgenic effects of this specific pesticide mixture, because the maternal toxicity limits the dose levels that can be studied in rats. However, as there are indications of anti-androgenic effects for several of the pesticides (MCPB, quinclamine, thiram and MCPB) further studies of these pesticides as well mixtures based on the potency for anti-androgenic effects appear warranted.

5. Conclusions and recommendations

The mixture study as well as the range-finding study showed clearly that the mixture of pesticides causing decreased birth weight, i.e. Cyromazine, MCPB, Pirimicarb, Quinoclamine, Thiram, and Ziram, caused decreased birth weight at dose levels markedly below NOAELs for this effect of the individual pesticides. The modelling of the expected mixture effects based on data for the single chemicals showed that dose-addition gave a good prediction of the observed mixture effect. Comparisons of the LOAEL for the mixture effect to the NOAEL used for deriving the Acceptable daily intake (ADI) indicate that some of the ADIs are not sufficiently low to protect against the mixture effects of the pesticides shown in this study. Also, for the rat dams mixture effects occurred at dose levels where the single pesticides show no effect on dams, i.e. at the observed NOAELs for maternal toxicity. Thus, the results imply that risk assessments based on NOAELs for single chemicals underestimate the risk and that there is a need to for cumulative risk assessment to take account of mixture effects and the potentially serious impact of mixed exposure on prenatal development and pregnancy. Observations of similar type of effect are recommended as the basis for grouping chemicals for cumulative risk assessment as the results of our study support findings from previous studies showing dose-additive mixture effects of substances that induce similar types of effects, but have dissimilar mechanisms of action. Last but not least, it is recommended when considering cumulative risk assessment to include all kinds of chemicals e.g. pesticides, industrial chemicals, environmental contaminants, as substances causing decreased birth weight exist within all of these chemicals classes and humans may be exposed to several of them simultaneously.

The decreased birth weight continued into adulthood of the animals which did not display catch-up growth to reach the same body weight as the controls. There was no effect on specific growth rate, food consumption, energy efficiency and oral glucose tolerance in any of the sexes or dosed groups. Also, no changes in the investigated organs involved in glucose metabolism (muscle and liver weights) were found and this was further confirmed by histological evaluations of pancreas and liver. At the high dose, PD 16 offspring showed a significantly increased weight of fatty tissue relative to the body weight and a tendency was still seen in males in adulthood. The biomarkers related to metabolic syndrome, obesity and diabetes showed no clear effects on the plasma levels of the measured hormones, except for a significant increase in plasma leptin levels in female offspring age 5-6 months and exposed to the highest mixture dose (mix-37.5 %). Some weak trends of dysregulation were discernible for some key regulator of metabolism and adipocyte differentiation (i.e. *Pparg* and orexigenic hormone encoding gene *Npy*) in exposed groups, but inconclusively so. Thus, the gene expression data show indications of being disturbed following pesticide exposure, but further experiments are needed to clarify if these effects are significant or not. Overall, no clear signs of metabolic syndrome or obesity were found in the offspring in adulthood. There are, however, indications that effects in the animals on these endpoints may not manifest themselves until the animals are aging. Hence, the very few effects observed at the age of 4-5 months in the present study do not rule out that effects could be seen later in life, e.g. in offspring between 1 and 1.5 years old.

The increased nipple retention in male offspring at the highest dose indicates anti-androgenic effect. However, the small magnitude of this effect taken together with the lack of effects on AGD and reproductive organ weights, indicate that marked anti-androgenic effects may only be seen at higher doses of the pesticide mixture. The mixture induced slight maternal toxicity at the highest dose in the main study and severe maternal toxicity at an only two times higher dose in the range-finding study. It is therefore most likely not possible to investigate, if there would be marked anti-androgenic effects of this specific pesticide mixture, because the maternal toxicity limits the dose levels that can be studied in rats. However, as there are indications of anti-androgenic effects for several of the pesticides (MCPB, quinclamine, thiram and MCPB) further studies of these pesticides as well mixtures based on the potency for anti-androgenic effects appear warranted.

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Appendix 1. Abbreviations

| | |
|---------------|---|
| AGD | Anogenital distance |
| ANOVA | Analysis of variance |
| BMD5 | Bench Mark Dose calculated to cause 5% decreased birth |
| weight | |
| BW | Body weight |
| DA | Dose addition, model for predicting mixture effect |
| DNEL | Derived no effect level |
| GD | Gestational day |
| Hrs | Hours |
| IA | Independent action, model for predicting mixture effect |
| LOAEL | Lowest observed adverse effect level |
| Mix | Mixture of pesticides |
| NOAEL | No observed adverse effect level |
| NOEL | No observed effect level |
| NR | Nipple retention |
| OECD | Organisation for Economic Co-operation and Development |
| PPAR γ | Peroxisome proliferator-activated receptor gamma |
| PD | Pup day |
| PND | Postnatal day |
| Rpm | Rounds per minute |
| SD | Sprague Dawley (rat strain) |
| SEM | Standard error of mean |
| STD | Standard deviation |
| TG | Test Guideline |
| Tox | Toxicity |

Combination effects of pesticides on birth weight and metabolic programming in rat offspring

Risk assessment of pesticides is generally based on the no observed adverse effect levels (NOAELs) for single compounds. However, humans are typically exposed to a mixture of several pesticides. The objectives of the project were to investigate whether a mixture of environmentally relevant pesticides cause decreased birth weights at dose levels below NOAELs for the individual pesticides in rats, to evaluate whether the mixture effect is best predicted by the independent action or the dose-addition model, to investigate the influences of developmental pesticide exposure on metabolic programming of the offspring using biomarkers for obesity and type 2 diabetes, and to give input for regulatory considerations.



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